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HCMV-INDUCED EPIGENETIC ALTERATIONS; A NOVEL PATHWAY TO CNS TUMORS?

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THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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Place: CMM lecture hall, L8:00, Karolinska University Hospital, Solna.

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I learned the best lessons in the hardest times.

I learned patience is the best faith.

I learned setbacks are delays, not failures.

Lastly,

I learned laughter is a miracle.

To My dearest

And

All cancer patients

Abstract

Aberrant DNA hypermethylation at CpG islands is a well-established phenomenon involved in transcriptional silencing of tumor suppressor genes. CpG island hypomethylation-mediated reactivation of oncogenes is also documented in several cancer types.

DNA methylation and histone modifications catalyzed by DNA methyltransferases (DNMTs), histone deacetylases (HDACs) and histone acetyltransferases (HATs), respectively, result in chromatin structure changes and altered gene regulation. Two categories of drugs, affecting histone acetylation and DNA methylation, are currently considered in epigenetic therapy of cancer. There is some evidence that synergistic effects of HDACi and DNMTi are achieved by their action on common targets, including DNA methyltransferase 1 (DNMT1). During the past decade, the human cytomegalovirus (HCMV) has been detected in several types of cancer including glioblastoma, the most lethal primary brain tumor with poor survival, and medulloblastoma, the most common malignant pediatric brain tumor. HCMV infection in glioblastoma is associated with poor outcome and antiviral treatment, was shown to restrict tumor growth and longer survival in these patients.

The aim of this thesis was to investigate events that may play a role in initiation and progression of cancer through epigenetic alteration with a special focus on HCMV infections. The main idea behind this approach is that, if epigenetic alterations and HCMV infection are highly prevalent in cancer, patients might benefit from effects caused by epigenetic and antiviral therapy.

In study I, changes in DNA methylation by the histone deacetylase inhibitor (HDACi), trichostatin A (TSA) was investigated in Hep3B cells. The effect of TSA on DNA methylation was studied at gene specific and global levels. Our results suggest that TSA causes genomic hypomethylation and leads to a decrease of nuclear DNMT1 protein levels by changing the DNA methyltransferases (DNMTs) mobility in the nucleus.

In study II, the impact of human cytomegalovirus infection on the host cell DNA methylation machinery was investigated. The results showed an alteration in the host cell DNA methylation machinery by the re-localization of DNMTs from the nucleus to cytoplasm. The data also suggested that DNA methylation influences cellular susceptibility to HCMV infection.

In study III, we explored the effect of HCMV infection on DNMT1 in medulloblastoma (MB) cells and tumor biopsies as well as human endothelial cells since blood vessels within MB tumors have shown to most often express HCMV proteins, and are important for tumorigenesis. The data demonstrated cytoplasmic localization of DNMT1 in HCMV infected MB and endothelial cells associated with expression of HCMV late gene UL55 (glycoprotein B, gB). Treatment of HCMV infected MB cells by the DNA methylation inhibitor, 5-azacytidine (5AZA), showed significant increased number of cells expressing viral protein. These data suggest that HCMV replication may benefit from inhibition of the host cell nuclear methylation machinery, a fact that may have consequences for using epigenetic drugs in cancer therapy.

In Study IV (Manuscript), the expression and localization of DNA methyltransferase 1 (DNMT1) and various HCMV proteins were assessed in glioblastoma (GBM) cells and patient tissue specimens. Moreover, the effect by 5AZA was analysed in the context of viral replication, proliferation and invasion ability of HCMV infected GBM cells.

Our data show that DNMT1 is localized to the extra-nuclear space of GBM cells expressing HCMV-gB proteins and in the cells of blood vessel wall within GBM tumors.

In tissue specimens, DNMT1 was shown to be expressed in the nucleus of tumor cells, but localized to the extra-nuclear /cytoplasmic space of cells lining blood vessel walls within the GBM tumors. 5AZA treatment of GBM cells lead to reduced proliferation of uninfected and HCMV infected cells and HCMV infected cells were shown to be vulnerable to 5AZA treatment, leading to a decreased invasion.

LIST OF SCIENTIFIC PAPERS

I. Mohsen Karimi Arzenani, **Atosa Esteki Zade**, Yu Ming, Susanne J. H. Vijverberg, Zhe Zhang, Zahidul Khan, Syed Sadique, Lorenz Kallenbach, LiFu Hu, Vladana Vukojevic and Tomas J. Ekström: **Genomic DNA hypomethylation by histone deacetylase inhibition implicates DNMT1 nuclear dynamics**. Molecular and cellular biology. 2011, Vol.32. p. 4149-4128

II. **Atosa Esteki-Zadeh***, Mohsen Karimi*, Klas Strååt, Ole Ammerpohl, Manuel Zeitelhofer, Maja Jagodic, Marjan Mehrab-Mohseni, Louise Sjöholm, Afsar Rahbar, Cecilia Söderberg-Nauclér and Tomas J. Ekström: **Human cytomegalovirus infection is sensitive to the host cell DNA methylation state and alters global DNA methylation capacity**. Epigenetics, 2012, Volume 7, p. 585-593.*Equal contribution.

III. **Atosa Estekizadeh**, Natalia Landázur, Jiri Bartek , Christian Beltoft Brøchner, Belghis Davoudi, Helle Broholm, Mohsen Karimi, Tomas J. Ekström and Afsar Rahbar: **Increased cytomegalovirus replication by 5-Azacytidine and viral-induced cytoplasmic expression of DNMT-1 in medulloblastoma and endothelial cells**. International Journal of Oncology, 2018, Volume 52, p.A1317-1327

IV. **Atosa Estekizadeh**, Natalia Landazuri, Belghis Davoudi, Giuseppe Stragliotto, Tomas J Ekström and Afsar Rahbar: **Nuclear exclusion of DNA Methyltransferase-1 and reduced invasion by 5-Azacytidine in Human Cytomegalovirus infected Glioblastoma cells**. (Manuscript)

RELATED PUBLICATION NOT INCLUDED IN THESIS

I. Yaiw KC, Mohammad AA, Costa H, Taher C, Badrnya S, Assinger A, Wilhelmi V, Ananthaseshan S, **Estekizadeh A**, Davoudi B, Ovchinnikova O, Shlyakhto E, Rafnsson A, Khan Z, Butler L, Rahbar A, Pernow J, Söderberg-Nauclér C: **Human Cytomegalovirus Up-Regulates Endothelin Receptor Type B: Implication for Vasculopathies?** Open Forum Infect Dis. 2015, Volume 2,

II. Sandén E, Dyberg C, Krona C, Gallo-Oller G, Olsen TK, Enríquez Pérez J, Wickström M, **Estekizadeh A**, Kool M, Visse E, Ekström TJ, Siesjö P, Johnsen JI, Darabi A.: **Establishment and characterization of an orthotopic patient-derived Group 3 medulloblastoma model for preclinical drug evaluation**. Scientific Reports, 2017, volume 7, Article number: 46366

III. Angelique Flöter Rådestad, **Atosa EstekiZadeh**, Leah Cui, Qurania Kostopoulou, Belghis Davoudi, Angelica Lindén Hirschberg, Joseph Carlson, Afsar Rahbar and Cecilia Söderberg-Naucler : **Impact of Human Cytomegalovirus Infection and Immune Response on Ovarian Cancer Survival** (Manuscript)

CONTENTS

LIST OF ABBREVIATIONS

5AZA	5-azacytidine
5mC	5-methylcytosine
BL	Burkitt's lymphoma
CGI	CpG island
CNS	Central nervous system
COX-2	Cyclooxygenase-2
CpG	Cytosine-phosphate-guanine
DNMT	DNA methyl transferase
E	Early
EBV	Epstein-Barr virus
EGFR	Epidermal growth factor receptor
ER	Endoplasmic reticulum
FDA	Food and Drug Administration
GBM	Glioblastoma
GCV	Ganciclovir
HAT	Histone acetylase
HBV	Hepatitis B viruses
HCMV	Human cytomegalovirus
HCV	Hepatitis C viruses
HDAC	Histone deacetylase
HDACi	Histone deacetylase inhibitors
HF	Human fibroblast
HHV-8	Human Herpesvirus-8
HMECs	Human mammary epithelial cells
HPC	Hematopoietic progenitor cell
HPV	Human Papillomavirus
HSPG	Heparin sulphate proteoglycan
HSV	Herpes Simplex virus
HTLV-1	Human T-cell lymphotropic virus-1

IBD	Inflammatory bowel disease
IE	Immediate Early
KSHV	Kaposi's Sarcoma-associated herpesvirus
LA	Late
LANA	Latency Associated Nuclear Antigen
LOI	Loss of imprinting
LUMA	Luminometric methylation assay
MB	Medulloblastoma
MBD	Methyl-CpG binding domain
MCMV	Murine cytomegalovirus
MCP	Capsid binding protein
MCPyV	Merkel Cell Polyomavirus
MECP2	Methyl CpG binding protein 2
MGMT	Methyl guanine methyl transferase
MIEP	Major immediate early promoter
ORF	Open reading frames
oriLyt	Origin of replication
PDGFR	Platelet derived growth factor receptor
RTT	Rett syndrome
SAHA	Suberoylanilide hydroxamic acid
SAM	S-adenosyl-l-methionine
TET	Ten-Eleven Translocation enzymes
TMZ	Temozolomide
TSA	Trichostatin A
UL	Unique long
US	Unique short
VEGF	Vascular endothelial growth factor
VGV	Valganciclovir
VPA	Valproic acid
VZV	Varicella Zoster Virus

1 INTRODUCTION

When I started this journey, the association between human cytomegalovirus (HCMV) and malignant gliomas (GBM), an incurable and aggressive primary brain tumor in adults, was shown by Cobbs et al. [1]. Later, between 2002 and 2012, several studies showed the presence of HCMV in other malignant tumors, such as colon cancer [2], breast cancer [3], prostatic carcinoma [4], mucoepidermoid carcinoma and salivary gland [5], as well as rhabdomyosarcomas [6]. Furthermore, Cecilia Söderberg-Nauclér's research group found longer survival in GBM patients, whose tumors had a low grade of HCMV infection in comparison to high grade, and the patients who received anti-viral treatment, valganciclovir, in addition to standard therapy. These data later got published in 2013 and 2015, respectively [7] and [8]. The standard treatment for GBM patients includes maximal surgical resection, followed by radiotherapy plus concomitant and maintenance temozolomide (TMZ) chemotherapy [9]. The TMZ is a DNA alkylating drug which results in methylation of guanine residues, inducing DNA damage and causing death of tumor cells [10].

Epigenetics is the principle of additional layers of genomic information for regulation of the primary DNA sequence, and include DNA methylation, and in its broader definition, histone modification, micro RNA, and chromatin remodeling. DNA methylation is heritable through cell divisions, reversible and dynamic, and is involved in a variety of diseases such as cancer [11-14], neurological disease [15] and viral latency and reactivation [16]. In the past decade, DNA methyltransferase inhibitors (DNMT inhibitors) and histone deacetylase inhibitors (HDAC inhibitors) have been recognized as potential antitumor drugs [17-19].

The epigenetic therapy that targets key proteins, like DNMTs and HDACs, has been clinically exploited in cancer treatment [20, 21]. In contrast to genetic errors in cancer, epigenetic aberrations can be modulated by chemical agents, and this makes them promising targets in cancer therapy. Among the identified epigenetic regulations in cancer, DNA methylation has been broadly investigated [22]. Due to this information, we started a project to study effects of epigenetic changes, specifically DNA methylation, associated to HCMV infection in CNS tumors. The method to study global genomic DNA methylation (LUMA), using a luminometric technology to quantitate methylation sensitive restriction digestions [23] had already been stablished in our lab when I started my project.

Below, I am describing my journey, my publications, and how this study may be of help in cancer treatment, and finally I mention all the people who were important in this journey.

1.1 EPGENETICS

To package and fit the entire length of around 2 meters of eukaryotic DNA in the nucleus of the mammalian cell, DNA is coiled around histone proteins building nucleosomes, and organized in a structure called chromatin. [24, 25]. Nucleosomes are the repeating units of chromatin consisting of histone octamers, on which 145-147 base pairs of DNA are wrapped. A histone octamer consists of two copies each of H2A, H2B, H3 and H4 which is compacting

DNA five- to ten-fold. This assembly of DNA and histone proteins are connected to the neighboring nucleosome by short DNA segments (~10–80 bp in length) called linker DNA and form chromatin fibers, with a diameter of ~10 nm, known as beads on a string [26, 27]. These fibers are then stabilized by binding of a fifth histone, H1, to each nucleosome and its adjacent linker, known as the 30 nm fibers. The condensation of these can be seen in the cell's metaphase and is defined as chromosomes. This structure is highly dynamic such that it has the potential to switch between a compact and accessible form of chromatin.

The compact form of the chromatin is inaccessible (heterochromatin) and therefore provide a poor template for biochemical reactions such as transcription, whereas the accessible form of chromatin, (euchromatin) is associated to active genes. This chromatin flexibility is associated to a wide range of histone modifications that are correlated with gene activity, DNA replication and DNA repair [27].

More than 100 different cell types in the human body have different fate and phenotype even though all of them carry identical genetic information and are derived from a single cell. The regulatory mechanisms that guide the cell to follow different developmental pathways and differentiate into different cells involve epigenetics [28]. In other words, epigenetics is "the study of heritable and potentially reversible properties in genome function, which are not directly dependent on the primary sequence of DNA". This means that some of the associated information with DNA, which is not embedded in the primary sequence, can also be inherited through cell division and transferred to daughter cells. During this process, the memory of a mother cells' function and phenotype can be retained in daughter cells [29]. Epigenetics is a very important level for interpretation and directing the function of the genome. Alterations of epigenetic states result in effects on DNA-protein interactions, in chromatin structure and compaction, and may also lead to significant alterations in gene expression [30] [31]. These epigenetic mechanisms ultimately play critical roles in various life process such as cell differentiation, growth, development, aging and immune response. Due to these functions, epigenetics can explain how environmental factors contribute to our individual phenotypes and explain susceptibility to certain disease such as cancer. In addition, epigenetic modifications can be considered as biomarkers for therapeutic treatments [32].

Epigenetic modifications are primarily comprised of DNA methylation, histone modification, micro RNA (miRNA)-mediated and long non-coding RNA (lncRNA)-mediated regulation [33]. One should be aware however, that only DNA methylation lives up to the original strict definition of epigenetic modification, i.e. heritability over cell divisions [34]. As the studies in this thesis have been focused on DNA methylation and histone modification, these are discussed in more detail in the following sections.

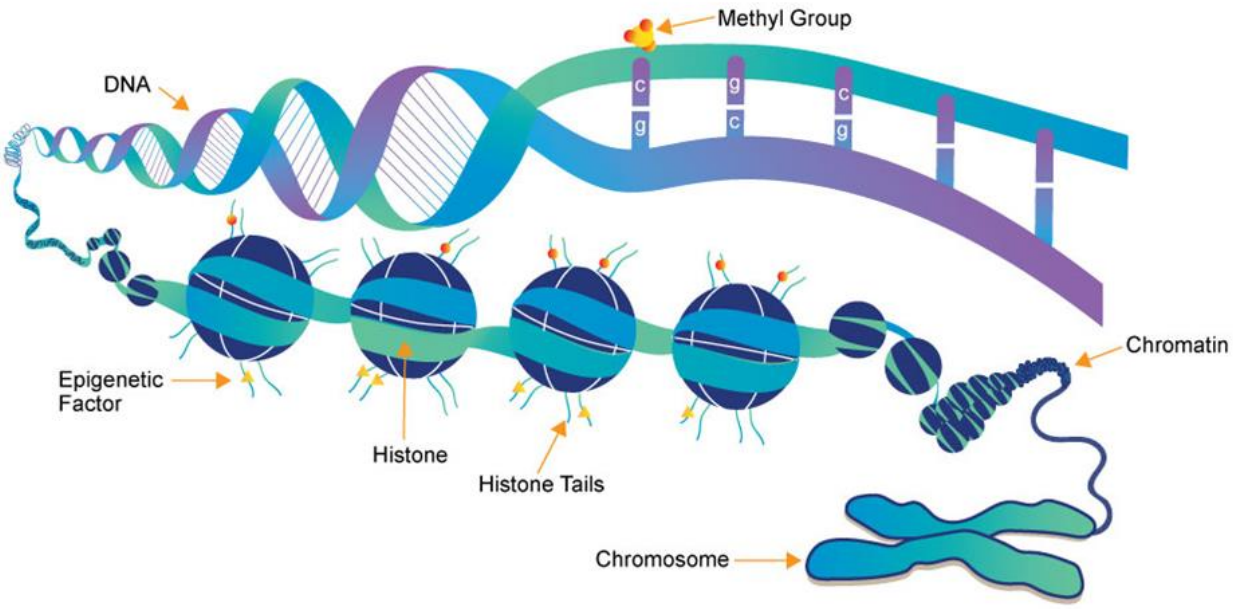

Fig 1. Chromatin structure, including histones and DNA, Adapted from (https://www.whatisepigenetics.com/fundamentals)

1.1.1 Histone modifications

Histones are nuclear proteins that perform critical functions in packaging and organizing DNA into structural units and to maintain gene regulation. According to the "histone code" hypothesis, the location and combination of histone tail marks has significance for eliciting downstream effects on cellular functions such as DNA replication, DNA repair, and mitosis, condensation, maintenance of centromeres and telomeres, and gene expression [35, 36]. Modification of chromatin can be divided into the adenosine triphosphate (ATP)-dependent chromatin-remodeling complexes, incorporation of histone variants and covalent histone modifications [37, 38]. Chromatin remodeling enzymes cooperates with site specific transcription factors and histone modification enzymes to move or to eject histones to be available for transcription factors to DNA [39]. Another mechanism which affect chromatin structure is incorporation of histone variants, of mainly H2A and H3. For example H2A can be replaced by H2AX (which is associated with DNA repair) and H3 can be replaced by H3.3 (associated with gene activation) or CENPA (associated with the centromer) [27]. The structure and function of chromatin are also controlled by covalent modification of histones. The subunit of histones (H2A, H2B, H3, and H4) contain N-terminal tails which are accessible to enzymes for chemical modifications which in turn affect the histone DNA interaction and modulate chromatin structure. Several different types of histone modifications are known, including acetylation, mono-, di-, or tri-methylation, phosphorylation, ubiquitination [35] [36, 40]. The combination of these modifications can produce millions of

different possibilities for each nucleosome. These enormous combinations can be read out by regulatory proteins and affect e.g. gene expression [41].

1.1.1.1 Histone Acetylation

Acetylation states of histones is the most studied histone posttranslational modification in the epigenome and it is associated with chromatin structure affecting gene activity [42]. Histone acetyltransferases (HATs) and histone deacetylases (HDACs) represent two enzyme classes that, respectively, catalyze the forward and backward reaction kinetics of lysine residue acetylation on specific protein substrates [43]. HATs catalyze acetylation by transferring of an acetyl group from acetyl-CoA to lysine residues in the histone tail. Thus, histone acetylation facilitates DNA accessibility and the interaction between DNA and other proteins including transcription factors, by reducing the attractive positive charge of histones to the negatively charged DNA backbone. HDACs, on the other hand, remove acetyl groups from histone tails leading to a greater attraction between the DNA backbone and the lysine positive charge, and package the chromatin into a more condensed structure. This prevents the accessibility to transcription factors and consequently inhibits transcription [43, 44]. To date, 18 human HDACs have been identified which are classified into four classes due to their localization in the nucleus or cytoplasm. HDAC1, 2 and 3 which are mainly located in the cell nucleus [45-47].

Regarding the involvement of the HAT-HDAC system in turnover of histone and transcription machinery regulation, the function of these two enzyme is one of the basic regulatory switches of gene expression. In addition to transcription regulation by HATs-HDACs, they are also postulated to modulate other chromatin-associated processes like replication, site-specific recombination and DNA repair, thereby playing a major role in modulating overall cellular fate [43]. A controlled balance between histone acetylation and deacetylation appears to be essential for normal cell growth [48]. Alterations in the structure or expression of HATs and HDACs has been reported in different cancers [49-52]. In most tumor cells, chromatin is hypoacetylated due to disruption of HATs activity or overexpression of HDACs. The hypoacetylation status of chromatin has been associated with low expression of either tumor suppressor genes or pro-apoptotic genes [53, 54]. It should be mentioned that both HATs and HDACs are not only modifying histone proteins, although their names suggest this. Many cellular proteins, e.g. the tumor suppressor protein p53 latency, are regulated by these enzymes [55]. The overexpression of HDACs, appears to silence tumor suppressor genes such as p21, a cell cycle progression inhibitor, resulting to tumor initiation and/or progression. It has been reported that the acetylation state of p53 by HATs/HDACs is part of the mechanisms that control the physiological activity/latency of p53 [55]

1.1.1.2 Histone deacetylase inhibitor

Perturbation of the balance between HDACs and HATs enzymes is, as mentioned above, often observed in human cancers, and inhibition of HDACs has developed as a new therapeutic strategy against cancer [56]. HDAC inhibitors are chemical agents inhibiting the activities of HDAC enzymes, causing an increase in the level of acetylated histones. This hyperacetylation in turn can promote the re-expression of silenced regulatory genes and may reverse the phenotype of cancer cells [57]. Pharmaceutical evaluations have shown that these drugs are able to selectively inducing growth arrest and apoptosis in different tumor cells while they have low toxicity in normal cells [57-59].

There are four main classes of HDAC inhibitors based on their distinct chemical structure, comprising hydroxamates (e.g. suberoylanilide hydroxamic acid (SAHA)), and hydroxamic acids, such as Trichostatin A (TSA), benzamides (e.g. MS-275), cyclic peptides (e.g. Romidepsin (FK-228)) and aliphatic acids (e.g. Valproic acid) [60]. The approval of three HDACIs (SAHA, FK-228 and recently PXD-101) by the Food and Drug Administration (FDA) has been followed by the development of more efficient and more potent HDACIs in cancer therapy [61, 62].

- Trichostatin A (TSA)

TSA is an antifungal antibiotic with cytostatic and differentiating properties in mammalian cell culture. As a HDAC inhibitor, its effect can be paramount by e.g. arrest the cell cycle and induce cell differentiation in culture [63, 64]. It has been reported that TSA is a potent inhibitor of tumor growth of human breast cancer cells [63]. Trichostatin A (TSA), has also been shown to reactivate DNA methylation-silenced genes even in the absence of DNMT inhibitors, suggesting a cross-talk between histone acetylation and DNA methylation [65]. In addition, it has been reported that TSA decreases DNMT3b mRNA levels in endometrial cell lines [66] and DNMT1 levels in Jurkat T cells [67].

- SAHA (Vorinostat)

SAHA has a similar structure as TSA and treatment of cells in culture with SAHA results in a marked hyperacetylation of histone H4 [68]. SAHA is used for the treatment of cutaneous T-cell lymphoma [69]. FK-228 (Romidepsin) is a cyclic peptide HDAC inhibitor, which acquired FDA approval for the treatment of cutaneous T-cell lymphoma and peripheral T-cell lymphoma [70].

Concerning gliomas, HDAC inhibitors such as TSA and Valproic acid (VPA) promote growth inhibition and apoptosis in different glioma cell lines [71]. VPA and other HDAC inhibitor drugs were examined in clinical trials and may be preferred among other drugs for prophylaxis and treatment of seizures in GBM patients [72, 73]. VPA has also been used for treatment of epilepsy and bipolar disorder [74].VPA causes hyperacetylation of the N-terminal tails of H3 and H4 *in vitr*o and *in vivo* and inhibits HDAC activity, probably by binding to the catalytic center and blocking the access of the substrate [75].This drug

influences differentiation and has antiproliferative effects by inducing the cyclin-dependent kinase inhibitor p21; p21 regulates cell-cycle progression (WAF1) [76].

1.1.2 DNA methylation

In 1948, Rollin Hotchkiss discovered modified cytosine in calf thymus using paper chromatography. He hypothesized that this modified cytosine was 5-methylcytosine (5mC) and naturally existed in DNA [77]. In 1978, it was reported that DNA methylation occurred almost exclusively on cytosine in a CpG dinucleotide context [78]. In the 1980s, several studies showed that DNA methylation was involved in gene regulation and cell differentiation [79, 80]. After these findings, several studies extensively showed the association between DNA methylation changes, in different diseases and carcinogenesis [81-86].

Many genomes contain various amounts of methylated bases, such as C5-methylcytosine (5mC), N4-methylcytosine (4mC) and N6-methyladenine (6mA) [87]. The main methylation in the eukaryotic genome is represented by 5mC [88, 89] and occurs on the 5 position of cytosine [90] [31]. Approximately 60% of gene promoters contain CpG islands (CGIs) and are largely unmethylated. CGIs are regions of more than 200 bases with a C+G content of at least 50% [91]. Some promoter-associated CGIs (approximately 6%) become methylated in a tissue-specific manner during early development or in differentiated tissues and result in silencing of downstream genes [91, 92].

In the human genome, containing approx. 56 million CpG sites, about 60-80% are methylated which is almost 4-6% of all cytosines [93]. These methylated CpGs are not randomly distributed but follow tissue and cell specific patterns [94, 95].

The process of establishment and maintenance of cytosine methylation is catalyzed by DNA methyltransferases (DNMTs) in the presence of the S-adenosylmethionine (AdoMet or SAM) cofactor as methyl group donor [96]. The human genome encodes five DNMTs; DNMT1, DNMT2, DNMT3a, DNMT3b and DNMT3L. DNMT1, DNMT3a and 3b are canonical methyltransferases that catalyze the addition of methyl group from SAM as a methyl donor to the cytosine within (usually) CpG dinucleotide of genomic DNA. DNMT2 and DNMT3L, in contrast, are non-canonical family members and DNMT3L has no catalytic activity [97].

DNMT1 prefers hemimethylated DNA over unmethylated DNA, and thus copy the existing methylation pattern of DNA during replication, which results in maintenance of the DNA methylation pattern through cell divisions [96, 98]. The DNMT3a and DNMT3b do not distinguish between unmethylated and hemimethylated DNA and mediate de novo methylation [99-101]. DNMT3L (DNMT-like protein) is catalytically inactive and does not have a functional catalytic domain [102].

Except for DNMT2, animal DNMT enzymes are usually multi domain proteins and contain two functional domain; an N-terminal regulatory domain and a conserved C-terminal domain required for catalysis. DNMT2 consists exclusively of the catalytic domain [103, 104].

DNMT2 is a highly conserved methyltransferase that specifically methylate tRNA instead of genomic DNA [105, 106]. In mammals, the regulatory domain guides DNMTs localization to chromatin by interacting with other proteins and DNA and regulates their intrinsic activity. The catalytic domain includes six different motifs involved in DNA binding and catalysis of methyl group transfer [102].

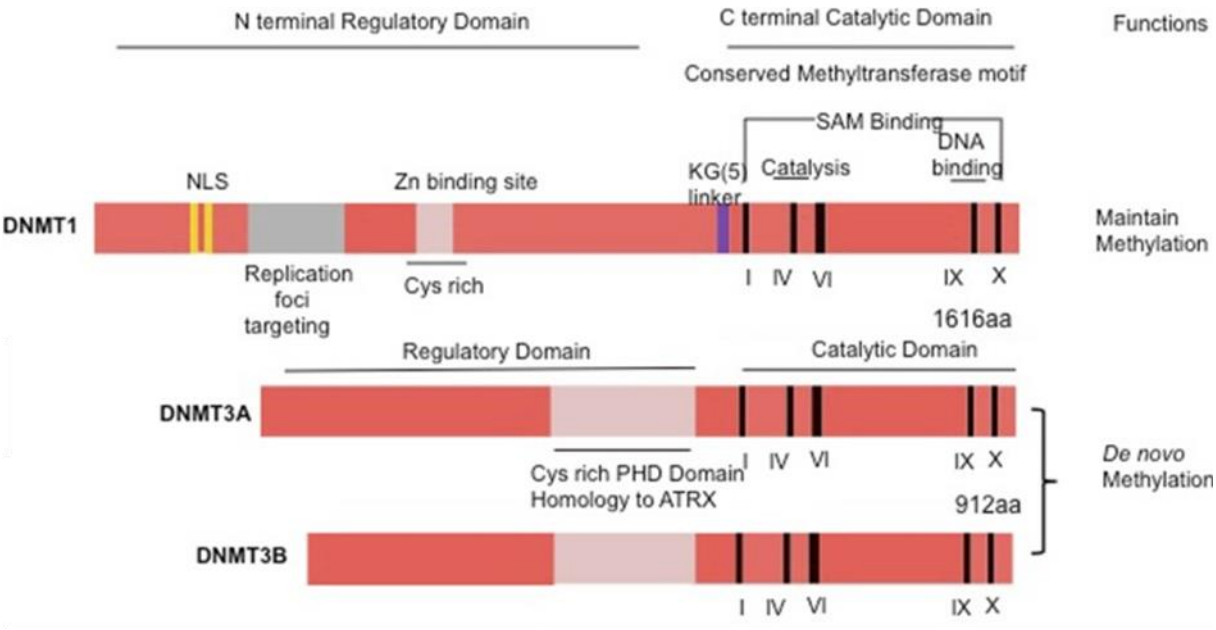

Fig 2. Schematic structure of mammalian DNMT family members, The N-terminal contains motifs of interaction with proteins or DNA. The C-terminal contains the conserved methyltransferases domains. PHD, plant homology domain, adapted from Dharmalingam Subramaniam et al. 2014

1.1.2.1 DNA methylation in different disease

DNA methylation has been shown to associate with many human diseases, including different types of cancer [107, 108], autoimmune diseases such as systemic lupus erythematosus [109], neurological disorders such as Alzheimer's and Parkinson's diseases [110] and psychological disorders. Also, it has been suggested that methylation can be involved in multifactorial diseases such as multiple sclerosis, cardiovascular disease and disease influenced by secondary factors such as sex differences and aging [111, 112]. Microarray studies in monozygotic twins and subsequently in unrelated individuals, confirmed a global decrease in DNA methylation with age, while site specific analysis showed an increase in variability of DNA methylation with age [113-115]. Emerging evidence has already shown the association between DNA methylation and inflammation in regulating immune pathways. For example, elevation of the cytokine IL-6 increases the expression of DNMT1 in T cells [116-119]. DNA methylation is involved in regulating differentiation, migration, and activation of T-cells [117, 118]. Another example is the

demethylation of the IL-2 promoter which results in upregulation of IL-2 and activation of T-cells [120].

SAM is thought to play an important role in brain development, reflecting its dominant and ubiquitous effects on cell biology in epigenetic psychiatry [121].

Three noticeable enzymes which catalyze DNA demethylation, are the Ten-Eleven Translocation enzymes (TETs), Tet1, Tet2, and Tet3 [122]. TET1, the most highly expressed TET in brain, is essential for the learning dependent accumulation of 5-hydroxymethylcytosine (5-hmC) and related, which underpins rapid behavioral adaptations based on epigenetic activation. TET1 has a role in neuronal activity-induced, region-specific, active DNA demethylation with concomitant gene expression changes [123]. In addition to catalytic activity, TET1 controls the levels of DNA methylation and thus regulates memory formation [124].

MECP2 encodes a methyl-DNA-binding protein that has been proposed to function as a transcriptional suppressor. MECP2 is a member of the MBD (methyl-CpG binding domain) proteins, is the highest expressed nuclear protein in the brain and known as a reader of DNA methylation. MECP2 binds to methylated DNA and mediates the molecular consequences of this epigenetic mark by recruiting other proteins involved in repression [125, 126].

Several studies showed that MECP2 is important for synapse maturation. The mechanism of MECP2 as a transcriptional repressor is not clear, although one study showed that it represses gene expression by binding to methylated CA dinucleotide sites within long genes [127-131]. Rett syndrome (RTT), a severe neurological disorder with feature of autism, is the result of disruption in the MECP2 gene [132]

1.1.2.2 DNA methylation in cancer

Cancer has traditionally been defined as a disease that originate from the accumulation of genetic mutations [133]. However, this definition has now been expanded to disruption of epigenetic regulatory mechanisms, which are prevalent in cancer [134, 135]. Both genetic mutations and epigenetic alterations ultimately involve abnormal gene expression. The genetic pathways of cancer include mutation of tumor suppressor and/or oncogenes which causes either gain or loss of gene function and/or abnormal gene expression. The epigenetic path of cancer involves chromatin structure, including DNA methylation, histone variants and modifications, nucleosome remodeling and small non-coding RNAs [136]. During tumor initiation and progression, the epigenome goes through multiple alterations, such as genome-wide loss of DNA methylation (hypomethylation), frequent increases in promoter methylation of CpG islands in tumor suppressor genes, changes in nucleosome occupancy and modification profiles. Thus, genetic and epigenetic alteration is now thought of as two separate mechanisms in tumorigenesis but whole exome sequencing of thousands of human cancers showed that these two mechanism are not separated events and they intertwine and take advantage of each other during carcinogenesis. Interestingly genetic mutations have the potential to disrupt the pattern of DNA methylation, histone modifications and nucleosome positioning. The other side of the coin is that epigenetic alteration can lead to mutations in

key genes and changing of gene expression [137], e.g. the methyl guanine methyl transferase gene (MGMT) whose gene product removes carcinogen-induced O6-methylguanine adducts from DNA and results in transition from G to A, can be hypermethylated in cancer. Hypermethylated MGMT may results in genetic mutation in critical genes such as p53 or KRAS [138, 139]. DNA methylation was the first epigenetic alteration identified in cancer [86]. Global DNA hypomethylation plays a major role in carcinogenesis and can occur at different genomic loci such as repetitive sequences, retrotransposons, CpG promoters, introns and gene desert; regions of the genome that are devoid of protein-coding genes [140]. DNA hypomethylation can induce genomic instability which is implicated in a variety of human cancer [141, 142] and activate proto-oncogenes [143]. In addition, hypomethylation of DNA can lead to the activation of growth-promoting genes, such as R-Ras and MAPSIN in gastric cancer, S-100 in colon cancer and MAGE (melanoma-associated antigen) in melanoma, and loss of imprinting (LOI) resulting in pediatric tumors associated with Beckwith-Wiedeman Syndrome (hepatoblastoma, Wilms tumor etc.) [144, 145].

Site specific DNA promoter hypermethylation is involved in carcinogenesis by silencing of tumor suppressor genes [81]. Promoter hypermethylation induce silencing of RUNX3 transcription factors in esophageal cancer, GATA-4 and GATA-5 transcription factors in colorectal and gastric cancers, leads to inactivation of their downstream target [146, 147]. Inactivation of DNA repair genes which enables cells to accumulate genetic lesions can lead to progression of cancer [148]. In addition to direct inactivation of tumor suppressor genes, DNA hypermethylation can also indirectly affect transcription factors and DNA repair genes. In support of developmental selectivity, DNMT1 and DNMT3a are expressed in both adult and the embryonic stages of the brain, whereas DNMT3b is just detectable during early neurogenesis [149]. There is a transition of mouse *de novo* methyltransferase expression from Dnmt3b to Dnmt3a during neural progenitor cell development [150] suggesting DNMTs as playing a particular role in neural function at specific times over neural maturation and development [151].

1.1.2.3 DNA methylation inhibitors

Aberrant DNA methylation in cancer and other diseases on the one hand and the reversible nature of epigenetic aberrations on the other hand, have led to making DNA methylation inhibitors as attractive targets for therapeutic intervention [136, 152].

Only two DNA methylation inhibitors have been FDA approved for cancer treatment. The cytosine analogs 5-azacytidine (5AZA, Vidaza®) and 5-aza-2'-deoxycytidine (decitabine, Dacogen®)) are demethylating agents successfully employed for myelodysplastic syndromes, acute myeloid leukemia and chronic myelomonocytic leukemia. These inhibitors are incorporated into the DNA and covalently trap DNMTs, resulting in global hypomethylation of the genome [153-156]. This hypomethylation or demethylation, can effect gene expression by re-activating tumor suppressor genes resulting in cancer cell death [157]. 5AZA can also independently change gene transcription by incorporating to RNA and alter mRNA stability. Additionally, 5AZA and decitabine cause DNA damage and activate the DNA damage

response in some cells; however, the nature of this DNA damage has not been fully characterized and is likely cell type-dependent [158]. Although these drugs are known to demethylate DNA, the link between demethylation-induced transcription and the therapeutic effects of these drugs is not well established [156]. Due to high toxicity, low specificity, chemical instability and the poor bioavailability of these drugs during cancer therapy, development of a new generation of nucleoside drugs is ongoing [154, 159]. The prodrug SGI-110 derived from decitabine is under investigation in phase 2 clinical trials for the treatment of myelodysplastic syndromes and acute myeloid leukemia [160, 161]. In epigenetic therapy, the combination of the hypomethylation agent 5AZA and HDAC inhibitors has a synergistic therapeutic effect on solid tumor and abolished chemo resistance in cancer cells [162, 163].

1.2 HUMAN CYTOMEGALOVIRUS (HCMV)

In 1898, nearly 120 years ago, Beijerinck, a Dutch microbiologist and botanist, was the first to suggest virus to be the incitant of the tobacco mosaic discoloration. The word virus originates from a Latin word meaning "venomous substance".

The first human virus described was the agent, which causes yellow fever transmitted by mosquitoes, discovered and reported in 1901. The agent was studied because many soldiers died of yellow fever in the Spanish-American war in 1980. Since then, there are many historical examples of mortality caused by viruses that led to a wealth of subsequent research to understanding the viral role in different diseases. Now we know viruses as infectious obligatory intracellular parasites comprising genetic material surrounded by a protein coat and/or membrane that can infect living cells [164] [165, 166].

1.2.1 Discovery and history of HCMV

In 1881, a German pathologist, Ribbert, observed enlarged cells in kidney specimen from stillborn infant who had died of syphilis like symptoms. He was unable to interpret this observation until he saw the report by Jesionek and Kiolemenoglou in 1904 [167]. Löwenstein described cytoplasmic and nuclear inclusions in these protozoal-like cells, followed by a clear halo in 1907 [168-170]. In the same year, Goodpasture and Talbot described these enlarged cells and used the term ‘cytomegalia’ although they did not have a clear explanation for the cause [171]. In 1925, Von Glahn and Pappenheimer shown that infected cells by herpesviruses contained inclusion bodies. They concluded that cytomegalic cells more likely were caused by a virus rather than protozoa [172]. Other researchers later supported the suggestion that a virus is the likely cause of the pathology of these cells, and this condition was termed a ‘generalised cytomegalic inclusion disease’ for the unknown viral aetiology of this pathology [173]. Minder later found the 199 nm particle in the clear halo around the intracellular inclusion of pancreatic cells in a case of cytomegalic inclusion disease by electron microscopy [174].

In the 1950, the virus was isolated from tissue cultures of human adenoid and salivary gland, and was called `salivary gland virus’ and thereafter, in 1960, Weller and colleagues proposed

the term `cytomegalovirus´ (CMV) [175, 176]. Propagation and isolation of CMV became possible once human cells were routinely grown in culture. Discovery of cell culture techniques enabled further development of the understanding of the nature of this virus, such as detection of virus protein, its life cycle and later its molecular pathogenesis [168, 177].

1.2.2 Epidemiology and transmission

Human cytomegalovirus (HCMV) infects 45%-100% of the world′s population, approximately 60% of adults in developed countries, and 100% in developing countries due to socio-economic circumstances [178, 179]. HCMV can be transmitted through different routes including horizontal close personal contact, vertical transmission from mother to child, also through other bodily fluids via breast feeding, urine, blood transfusion, stem cell and organ transplantation, and sexual contact [179]. A primary infection can be acquired in a fetus, a neonate, a toddler, a child or an adult that is generally mild or asymptomatic in immunocompetent hosts [180-182]. Even though acute symptoms are rare in immunocompetent, healthy hosts can have symptoms including headache, fever, a sore throat, malaise, lethargy, splenomegaly, lymphadenopathy, upper respiratory tract infection or more severe mononucleosis [183]. In contrast to healthy people, HCMV can cause life-threatening disease in immunocompromised patients. HCMV can induce distinct end-organ diseases in patients with HIV/AIDS, transplant recipients treated with immune suppressants, premature infants and individuals with a suppressed immune system [180, 184].

HCMV is the most important infectious agent associated with acute and chronic rejection in transplant patients, cardiovascular diseases [185], bacterial [186]and fungal infections [187], post-transplant diabetes [85, 188, 189] and significant morbidity and mortality following organ transplantation [190, 191]

Recent meta-analysis reveal a significant connection between HCMV and increasing risk of atherosclerosis and vascular disease [192, 193]and it has been shown that HCMV infection is associated with an increased risk for inflammatory bowel disease (IBD) [194]and steroid-resistant IBD [195]. During the last years, emerging evidence suggest that human malignancies arising from different tissues can be attributed to HCMV [1-4, 6, 196, 197].

1.2.3 Herpesviridae

Herpesviriedae is a large family of viruses with linear double stranded DNA (100-225kbp), which can infect most vertebrates, from fish to mammals [198]. According to phylogeny analysis, the herpesviridae family emerged 180-220 million years ago, probably before generating the mammalian line, 80 to 60 million years ago [199] .

Among hundreds of known herpesviruses, at least eight species can infect humans, and are classified into three subfamilies, alpha, beta and gamma, according to differences in their biological properties such as host cell tropism, latency and their different clinical manifestations [199]. Alpha herpesviridae includes, HSV1, HSV2 (Herpes Simplex virus 1, 2) and VZV (Varicela Zoster Virus) and have a variable host range. HSV-1 and -2 and VZV

remain latent in neuronal cells of dorsal root ganglia [200, 201]. Approximately 90% of the world´s human population is infected with one or both HSV and suffer from genital and or oral lesions, encephalitis, aseptic meningitis and conjunctivitis. Except for VZV, there is no vaccine to prevent herpesviruses [202, 203].

Beta herpesviruses, includes human cytomegalovirus (HCMV) and Roseolovirus (Human herpesvirus type 6 and 7). They have restricted human host range, and long replication cycle with quite slow progression in culture [198, 203-205]. Human cytomegalovirus from this subfamily can cause congenital infection which may lead to intrauterine fetus death. However, most of the children with congenital HCMV infection are asymptomatic at birth, HCMV infection in new born baby is the most common cause of congenital abnormalities, occurring in 0.2% to 2.5% of all births [206]. The HCMV infection most commonly causes hearing loss in new born, but mental retardation and visual impairment are also observe. Other clinical manifestations include growth retardation, seizures, lethargy, microcephaly, thrombocytopenia and anemia [206]

Gamma herpesvirinae viruses are oncogenic viruses and includes Epstein-Barr virus (EBV) and Rhadinovirus (Human herpesvirus type 8), which is also named Kaposi's Sarcoma-associated herpesvirus (KSHV) as the endothelial cells in vessels are infected by HHV8. All members of this group replicate in lymphoblastoid cells, and also cause lytic infections in some types of epithelioid and fibroblast cells. Viruses in this group are usually T- or B-lymphocyte specific, and establish latency in these cells [203-205]. EBV from this sub family is linked to the etiology of several lymphoid and epithelial malignancies such as nasopharyngeal carcinoma, Burkitt's lymphoma (BL), post-transplant lymphomas and gastric carcinomas [207].

1.2.4 HCMV structure

HCMV is the largest of herpesviridae and a typical herpesvirus, with a virion size varying from 200 to 300 nm. The virion, (virus particle), consists of an icosahedral nucleocapsid made of 162 capsomers surrounded by an unstructured proteinaceous layer called the tegument. The tegument is enclosed by a lipid layer envelope derived from the host cell membrane and containing viral glycoproteins that are involved in attachment and entry into cells [208, 209].

1.2.4.1 Genome

HCMV genome consists of approximately 235 kbp, containing 252 open reading frames (ORFs) which are believed to encode 180 proteins. However, one recent study suggests that HCMV may encode 751 proteins indicating that HCMV may be far more complex than previously believed even though the functions of most of these proteins are still unknown [210].

The HCMV double stranded DNA genome, composed of unique long (UL) and unique short (US) domains, which are flanked on one end by terminal repeated sequences (TRL and TRS)

and on the other end by internal repeats (IRL and IRS) [211].The genome of HCMV contains only one origin of replication (oriLyt), in the UL sequence [65, 209, 212, 213]. Recombination between the terminal repeats and the internal inverted repeats leads to inversion of the UL and US sequences which results in the formation of different viral isomers during replication of herpesviruses [214].

The HCMV genome, exists as an episome during infection. The episome is associated with nucleosomes in the infected host cells but the viral DNA lacks histones when it is encapsidated into the virion [215]. It was reported that the host cell nucleosome deposition machinery, targets HCMV DNA in infected cells, resulting in a stepwise and dynamic viral chromatin assembly [216].

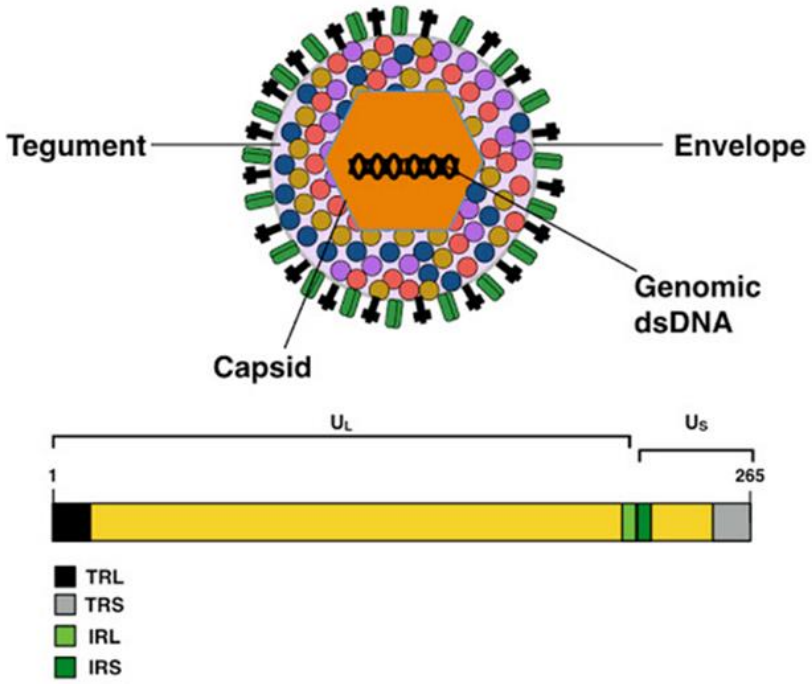

Fig 3: HCMV virion and outline of genome , Adopted from:
http://virologytidbits.blogspot.se/2016/01/human-cytomegalovirus-hcmv-and.html

1.2.4.2 Capsid

The capsid or nucleocapsid is the innermost core layer of a virion particle which contains and protects the viral genome. The nucleocapsid shows underlying icosahedral symmetry and made of 162 capsomers. The capsid is composed of at least five virus core proteins including, Major Capsid Protein (UL86), Minor Capsid Protein (UL85), Smallest Capsid Protein (SCP, UL48- 49), Assembly protein (Fragments of UL80) and Minor Capsid Binding Protein (MCP, UL46)[217].

Based on capsid assembly during viral replication, there are three types of HCMV capsid present in the nucleus of infected cells, the A-capsid which is only a capsid shell, the B-capsid which is a capsid shell with assembled proteins and the C-capsid which is a mature capsid including the viral genome and immune-evasion [218].

1.2.4.3 Tegument

The nucleocapsid is enclosed by the tegument which is an amorph protein coat for the capsid and contains the majority of virion proteins [219]. Most of these tegument proteins are phosphorylated, thus often designated with a prefix pp and are highly immunogenic [220] [221]. The most studied tegument proteins are pp65/ppUL83, pp71/ppUL82, pp150/pUL32 and pp28/pUL99. In addition, tegument also contains additional proteins in small amounts and some cellular and viral RNA. These proteins play major roles during virus entry, intracellular capsid transportation and assembly [222]. Many of host encoded proteins are also found in the viral tegument [220].

1.2.4.4 Envelope

The viral envelope is a lipid bilayer which surrounds the tegumented capsid, contains both viral glycoprotein and host cell proteins. It interacts with the host cell membrane on target cells and thereby plays a major role during virus attachment and entry. The phospholipid envelope contains several virus encoded glycoproteins, including gpUL55 (gB), gpUL73 (gN), gpUL74 (gO), gpUL75 (gH), UL100 (gM), gpUL115 (gL) and pentameric complex consisting of gL, gH and UL128-131. These glycoproteins play essential roles in virus entry into host cells, cell-to-cell spread, and virion maturation [223, 224].

1.2.5 HCMV entry

In general terms, the process of enveloped virus's entry requires an attachment to the host cell surface, including interaction between virus particle and a host cell receptor, internalization of the particle or fusion of the viral envelope with the cell membrane of the host cells.

Although the cellular targets of HCMV infection is not completely understood, the extensive expression of cell surface receptors in permissive cells, include, proteoglycans, cellular integrins (specifically αvβ3), and epidermal growth factor receptor (EGFR), β2 microglobulin, annexin II, aminopeptidase (CD13) and platelet derived growth factor receptor-α (PDGFR-α), suggest that multiple cell types support viral entry [225-228].

HCMV encodes at least 57 glycoproteins, 14 of which have been biochemically proven to be the structural components of the virion [221]. The main HCMV glycoprotein complex which have important functions during attachment and entry of the virus into the host cell including, gB, gH, gL ,gM, gN, and gO are also involved in immune evasion and determination of host cell tropism [229-231]. Glycoprotein B (gB) is one of the most abundant HCMV glycoproteins. As the major viral envelope constituent, HCMV-gB is involved in attachment to the cells via binding to cellular receptors including heparin sulphate proteoglycan (HSPG), integrin (αVβ3) and epidermal growth factor receptor (EGFR), annexin II and Toll-like

receptor 2, to promote the entry process [226, 227, 232-235]. Glycoprotein B is also involved in viral fusion and essential for the infection of all types of target cells [236]. In essence, gB is an attractive target for inclusion in a human vaccine and a major focus of experimental vaccination strategies [237]. The heparan sulphate proteoglycan molecule allows the HCMV particle to attach closer to the cell membrane where viral glycoproteins gB and gH/gL bind to specific receptors for cell-to-cell contact and for sufficient viral entry [238-240]. In addition, HCMV-gH act as a co-receptor by interacting with integrin αVβ3 [238, 241]. HCMV gH and gL associate with a large, heavily glycosylated viral glycoprotein gO and facilitate HCMV entry into fibroblasts independent from pH in cell culture [242].

Several other viral protein complexes are also involved in the viral entry process, including a homodimer of gB called gC-I, and a hetero dimer composed of gM and gN that form gC-II and a heterotrimer of gH, gL and gO that forms the gC-III complex [242-244]. It has already been shown that there is a number of genes from a laboratory strain HCMV which are important for entry into endothelial and epithelial cells [245, 246]. Three of these proteins, UL128, UL130, and UL131, bind gH/gL to form a quinary complex that functions in entry into these cells [224]. These accessory proteins may stimulate a transient interaction between gH/gL and gB to induce membrane fusion [247]. HCMV entry into endothelial cells depends on gB, gH/gL/gO glycoprotein in addition to envelope pentamer complex (PC) composed of gH, gL, UL128, UL130, and UL131A[238, 246, 248-253].

The role of HCMV gH/gL has not yet been completely defined but it has been shown to promote efficiency of fusion via selected entry pathways important for cellular tropism [254]. In conclusion, over the past years it has been recognized that there are distinct routes of HCMV entry into fibroblasts, endothelial and epithelial cells depending on an intricate interplay of different sets of envelope glycoprotein complexes. HCMV has two different entry routes in different cell types, in fibroblasts, viral entry into the target cells requires glycoprotein complexes composed of gB and gH/gL/gO and in endothelial and epithelial cells, viral entry requires gB, gH/gL/gO glycoprotein in addition to envelope pentamer complex (PC) composed of gH, gL, UL128, UL130, and UL131A [238, 246, 248-253].

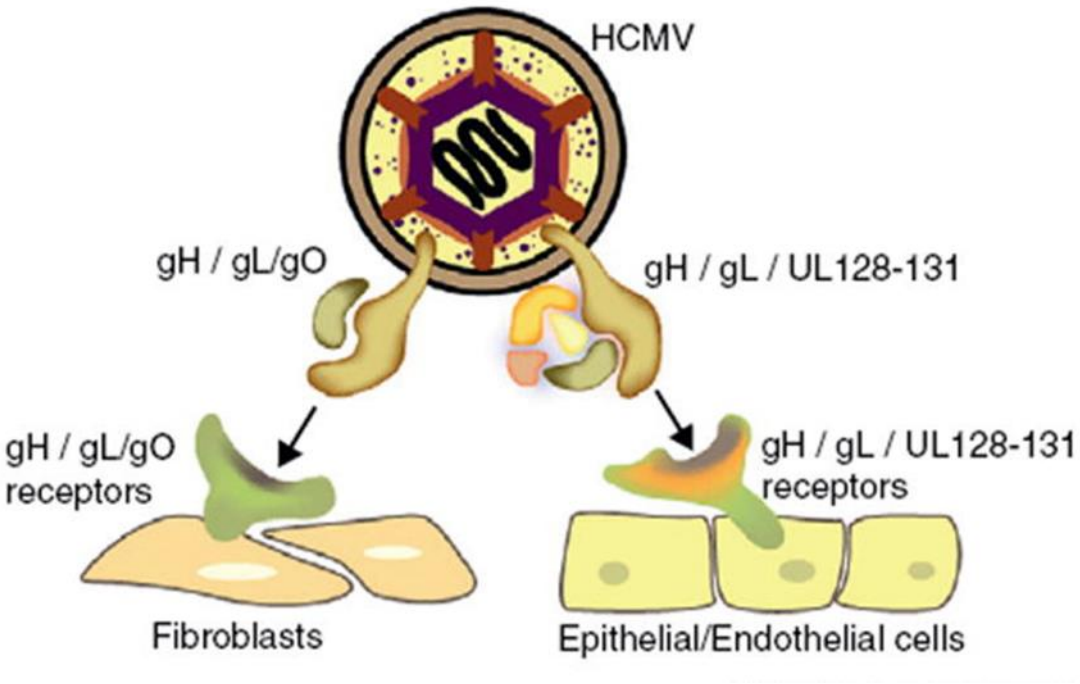

Fig 4: Human cytomegalovirus entry into cells, Figure from Adam L Vanarsdall and David C Johnson, 2012.

1.2.6 Viral replication

After virus fusion with the cell membrane, the HCMV nucleocapsid is deposited into the cytoplasm and viral DNA delivered into the host cell nucleus. Following infection, the viral genome is transcribed in a highly regulated sequence, resulting in the serial transcription of three different classes of the protein; immediate-early (IE), early (E), and late (L) [255] HCMV-IE proteins are the most abundant proteins which are expressed in the first step of viral replication cycle; it takes around 4 hours following infection, while the complete replication of HCMV yielding complete viral progeny, requires 48-72 hours. Expression of IE proteins regulates the subsequent expression of other viral genes (E and L genes), by acting as trans-activators or auto-stimulators. E proteins mostly affect the transcriptional and replication machinery for the viral protein production and interact with the host DNA. The L proteins are mainly structural components (capsid, tegument and envelope proteins) of the virions and also include proteins essential for virion assembly and egress [255, 256].

HCMV encodes its own proteins for the replication machinery such as a DNA polymerase (UL54) and DNA primase (UL70) to sustain an efficient production of new virus progeny [257].

The HCMV DNA replication is a bidirectional process followed by a rolling circle mechanism which originates from a cis-acting lytic origin of DNA replication (OriLyt) element to generate viral DNA molecules to be incorporated into new virus particles [258, 259].

1.2.7 Viral assembly

When the replication cycle is completed, viral DNA is inserted into the preformed capsids and become a mature capsid. This nucleocapsid is exported through various cellular compartments (ER and Golgi complex) where it acquires the tegument and envelope [217]. It is unclear how tegumentation takes place, but as no capsids in the nucleus are tegumented, while all are in the cytoplasm, the tegumentation must take place during or rapidly after nuclear egress. The tegumented particle then egress to cytoplasmic vacuoles for final envelopment [260]. The virions are released/transferred through cell lysis or cell-to-cell contact [217]. It has already been shown that Golgi-derived secretory vacuoles containing mature virus particles also fuse with the plasma membrane resulting in release of new infectious viral particles from infected cells [260].

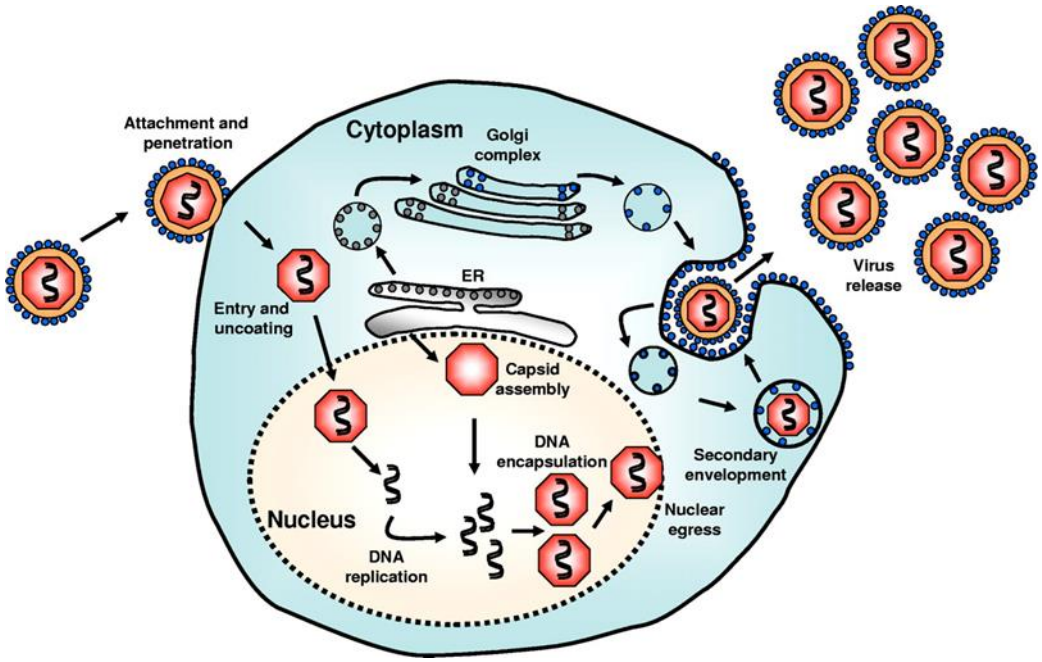

Fig 5: Life cycle of HCMV in a human cell, Figure from Crough T and Khanna R., 2009

1.2.8 Latency and reactivation

Viral latency is a central strategy by which herpesviruses ensure their life-long persistence without destroying the host. Latency is an interaction between virus and host cell that allows a latent state which can change into an initiation of productive replication in response to host cues. However a number of fundamental questions about the nature of HCMV persistence and the mechanisms regulating this latency and reactivation remains poorly understood [261]

During primary infection, HCMV infects various cell types including macrophages, endothelial cells, epithelial cells, fibroblasts, stromal cells, neuronal cells, smooth muscle cells, microglial cells and hepatocytes [225, 262-264]. While some cell types support viral

replication, other cell types such as hematopoietic progenitor cells (HPCs) and cells of the myeloid lineage (e.g. CD14+ monocytes), harbor viral genomes in the absence of active replication, providing latent reservoirs for the virus [265, 266]. Smooth muscle cells and endothelial cells have also been implicated as sites of HCMV persistence and latency. It is believed that the replication of HCMV in these two cell types play an important role in enabling the virus to maintain a life-long infection within the host [267-269].

During latency, the viral genome is maintained with a limited latency-associated transcription program which does not result in the production of viral particles [270]. It is now relatively well established that the differentiation-dependent reactivation of HCMV in the myeloid lineage is associated with changes in posttranslational modifications of histones around the major HCMV lytic promoter, namely, the major immediate early promoter (MIEP). These changes result in major changes in MIEP gene products and initiate the lytic transcription program and the production of infectious virions [215, 270-272].

It has also been shown that the establishment of murine cytomegalovirus (MCMV) latency, in vivo, is associated with repression of immediate early gene expression, deacetylation of histones bound to MIEP and changes in methylation pattern of histones bound to MIEP, and also recruitment of cellular repressors of transcription to the MIEP [273, 274].

In vitro studies showed that, latently infected monocytes have to differentiate into macrophages or dendritic cells in order to induce reactivation of HCMV after which the virus then enters a lytic phase [270, 275]. It is becoming apparent that the reactivation process is orchestrated by both cellular and viral factors, extracellular signals resulting in epigenetics alterations [276-279].

1.2.9 HCMV different strains

The first complete HCMV genome sequence, derived from the highly passaged laboratory strain AD169, was published almost 28 years ago, in 1990 [280, 281]. Since then, many researchers have sequenced various strains of HCMV [282]. The structure and genetic content of wild type HCMV have not been well studied, however. Most studies were done on HCMV strains which are extensively passaged in human fibroblast cells, even those performed to compare the complexity and variations of HCMV genome sequences. A low passage clinical strain, denoted Merlin, is today considered as the reference genome (NCBI GenBank accession NC_006273.2). According to clinical data, different HCMV strains exhibit different levels of virulence depending on their passage history in cell culture and competencies in tropism for endothelial and epithelial cells. These differences could be attributed to the possible loss of genetic information in these viral strains during propagation in cells [283].

AD169 and Towne are the most studied HCMV strains originating from wild type virus and are extensively passaged in human fibroblasts (HF). These two attenuated viral strains were developed as vaccine candidates and used in clinical trials [284]. In contrast, the Toledo strain

which is a low-passaged HCMV strain, has produced clinically apparent disease when administered to healthy adult volunteers [285].

Analyses have shown that almost all strains passaged in fibroblasts, have lost all or part of the UL128L region and genes of the RL11 family, indicating a possible role in cell tropism for these gene products. UL128L consists of the genes UL128, UL130, and UL131A and its products form a complex with the viral glycoproteins gH and gL. While this complex is unessential for growth in fibroblast it is essential for endothelial and epithelial cell tropism [248, 286]. The RL11 gene family contains 14 genes at the 5' end of the UL region (RL5A, RL6, RL11-UL1, UL4 -UL11) that are essential for growth in fibroblasts and are functionally poorly characterized [287-289].

HCMV strains, Towne and AD169 have substantial deletions (13 and 15 kb respectively) in the long unique region (UL) of the viral genome, combined with a compensating expansion of the long terminal repeats, TRL/IRL [290]and has several different additional mutations, which have important functional consequences for viral cell tropism and infection [291-293]. AD169 has open reading frame (ORF) disrupting mutations in genes RL5A, RL13, UL36, and UL131A; Towne is affected in genes RL13, UL1, UL40, UL130, US1, and US9 [287, 291, 294-296]. Even the low-passage strain Toledo is mutated in genes RL13, UL9 and UL128 genes [297].

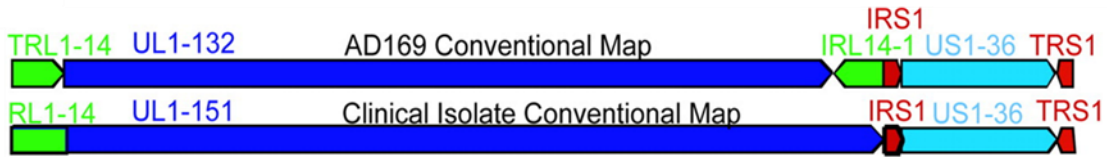

Fig 6: HCMV ORF organization, adapted from Eain Murphy, et al. 2003

1.2.10 Treatment

In spite of examining a variety of vaccine strategies, there is not yet any available vaccination against HCMV [298]. To date, five licensed antiviral drugs are used for the prevention and/or treatment of HCMV infections: ganciclovir, valganciclovir, foscarnet, cidofovir and fomivirsen [299].

- Ganciclovir and Valganciclovir

Ganciclovir (GCV) was the first antiviral drug available for HCMV treatment. GCV is a pro-drug and an analogue of guanosine that is first monophosphorylated by viral UL97 kinase and then become di- and triphosphorylated by a cellular kinase. This ensures that only infected cells are treated. This triphosphorylated guanosine analogue incorporate into the viral DNA chain and inhibits viral DNA polymerase and viral DNA synthesis. Ganciclovir is available in oral and intraocular formulations [299]. Valganciclovir (VGV) is a pro-pro-drug and available as an enteral formulation that rapidly metabolize to the active form, ganciclovir, in

the liver and intestinal wall [300, 301]. GCV resistance, which commonly occurs after solid organ transplantation, can be caused by mutation in the viral protein UL97, phosphotransferase, genes or the UL54 (viral DNA polymerase gene). More than 90% of isolates contain one or more mutations in the UL97 gene. The major side effect of GCV involves hematological abnormalities, particularly neutropenia but also toxicity effect on the liver and kidney [299].

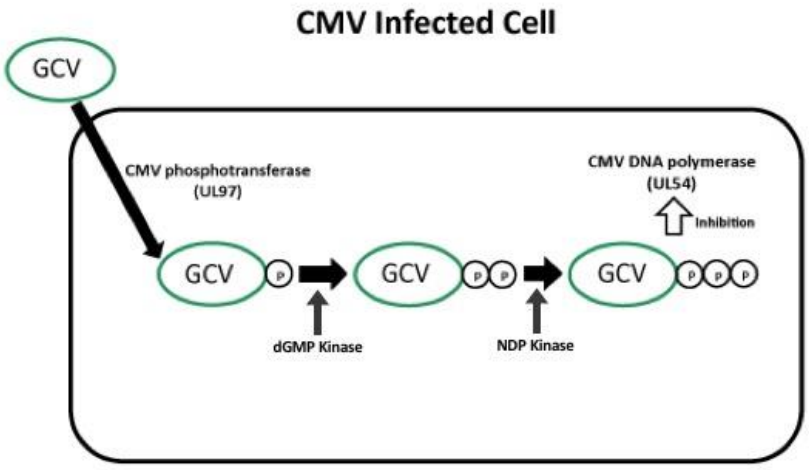

Fig 7: Mechanism of Ganciclovir function in CMV infection, adapted from https://www.google.se/search

- Foscarnet

Foscarnet is used for those who cannot be treated with GCV due to dose limiting neutropenia or leucopenia, or due to resistance to GCV [302]. It has been suggested that this drug selectively inhibits the pyrophosphate binding site on viral DNA polymerase and inhibits its function and does not require cellular or viral kinases to be activated [303].

- Cidofovir

This drug is used only as a second line therapy, due to its side effect. Cidofovir requires cellular kinases to be activated and causes premature termination in viral DNA synthesis and inhibits viral DNA polymerase [304, 305].

- Fomivirsen

Fomivirsen can block all classes of HCMV gene expression as it is an antisense oligonucleotide inhibitor of HCMV-IE mRNA. This drug is used for treating HCMV retinitis in immunocompromised patients, mostly HIV patients [303]. It has a half-life of

approximately 55 hours, which allows infrequent dosage and due to its intraocular administration, it has no systemic effect during treatment [306].

- Novel inhibitors of HCMV

Although drugs currently available for the treatment of HCMV disease has proven successful in immunocompromised patients, their use is limited. This limitation is due to their side effects, toxicity, poor oral bioavailability, modest efficacy and the development of drug resistance. In some cases, such as pregnant women or congenitally infected patients, these drugs should not be used due to their teratogenic effects. Therefore, there is a need to develop new compounds against HCMV diseases with alternative mechanisms of action e.g. the disruption of other steps in viral propagation such as attachment, entry, viral gene expression and function [303, 307].

Currently, there are some new drugs in preclinical studies:

- Maribivir is an inhibitor of HCMV UL97 kinase. UL97 is an important molecule that digest the nuclear lamina, and inhibition of UL97 prevents the maturation of capsid required for the egression from the nucleus to cytoplasm [308]. It has undergone phase-II trial and it has failed in phase-III. Further, Maribivir cannot be used with GCV due to its antagonizing effect on the drug [309, 310].

- Brincidofavir is a lipid prodrug of Cidofovir. Even though it has no renal toxicity as compared to Cidofovir, it has certain gastrointestinal toxicity, manifested as diarrhea [311].

- Letermovir is a unique antiviral drug which inhibits the viral terminase enzyme. Packaging of herpesviral DNA is believed to be mediated by the terminase enzyme which includes cleavage of viral DNA and packaging into the capsid. Inhibition of viral terminase by Letermovir prevents packaging of viral capsid containing viral DNA [312].

A recent publication identified the endothelin B receptor antagonist, Macitentan, which could be a potential future treatment option for HCMV infection [313]. Currently, this drug is used for treatment of patients with pulmonary hypertension and blocks HCMV infection *in vitro* by interfering with receptor binding, signaling and transcription of HCMV IE proteins [314].

One study showed that a potent and selective small molecule, CF102, can inhibit gB mediated HCMV virion fusion and cell-cell spread. CF102 acts very early after infection in the replication cycle and inhibits virion envelope fusion with cell plasma membrane. CF102 is not being developed clinically due to *in vivo* stability and metabolic issues [235].

1.2.11 HCMV and cancer

1.2.11.1 Oncoviruses

Approximately 12% of all human cancers are caused by oncoviruses [315]. Although a large number of the world's population harbors at least one of these oncoviruses, these infections rarely result in cancer [316]. Oncoviruses are classified as direct and indirect carcinogenesis,

even some overlap exist in between [317]. Direct carcinogenesis includes viral oncogenes that directly contribute to transformation and cellular neoplasticity, whereas indirect carcinogens cause chronic inflammation and subsequent oncogenic transformation through a number of steps [318]. In fact, oncoviruses encode gene products that can induce cellular transformation under certain circumstances [319-321]. Currently, there are seven recognized human oncoviruses which include Epstein - Barr virus (EBV), Human Papillomavirus (HPV), Hepatitis B and C viruses (HBV and HCV), Human T-cell lymphotropic virus-1 (HTLV-1), Human Herpesvirus-8 (HHV-8), and Merkel Cell Polyomavirus (MCPyV) [322]. EBV is linked to the etiology of several different lymphoid and epithelial malignancies, such as nasopharyngeal carcinoma, Burkitt's lymphoma (BL), post-transplant lymphomas and gastric carcinomas [207].

Many oral and other anogenital malignancies as well as over 99% of all cervical cancers are associated with high-risk HPV infections (HPV 16-18)[323].

Hepatitis B and C viruses (HBV and HCV) both are hepatotropic viruses known to be a risk factors for hepatocellular carcinoma (HCC) [324].

Some other viruses are connected to different neoplastic diseases, e.g. Human T-cell lymphotropic virus-1 (HTLV-1) are linked to adult T-cell leukemia (ATL) [325], Human Herpesvirus-8 (HHV-8) to Kaposi's sarcoma (KS) and primary effusion lymphoma [207], and Merkel Cell Polyomavirus (MCPyV) are linked Merkel cell carcinoma (MCC) which is an extremely rare and aggressive cutaneous cancer [322].

Although all these viruses do not belong to the same family of viruses they have the ability to infect and remain in the host cell without completely destroying it. They establish persistent and long term infections, and importantly, have strategies to evade the host immune system [326].

1.2.11.2 Prevalence of HCMV in cancer

The concept of a role of HCMV in cancer is not new. The presence of HCMV in prostate cancer was reported by Fred Rapp's group in the 1970s. They isolated a HCMV strain from tumor that was oncogenic *in vitro* and in immuno-deficient mice [327]. Later and over the previous years, HCMV proteins and nucleic acids have frequently been detected in tissue specimens from patients with cancers of different origin, including colon [2], breast [3], prostate [4], mucoepidermoid salivary gland tumors [5], medulloblastoma [196], glioblastoma [1, 328, 329], neuroblastoma [330] and rhabdomyosarcoma [6]as well as metastatic tumors [331, 332]. Although HCMV proteins were mainly detected in tumor cells, and sometimes in endothelial or inflammatory cells adjacent to the tumor, no or little of HCMV IE proteins were found in the healthy tissue surrounding the tumor tissue [2].

One recent study isolated a clinical HCMV strain denoted HCMV-DB [333] which can transform human mammary epithelial cells (HMECs) *in vitro* and *in vivo*. Infection of HMECs cells with HCMV-DB was followed by the inactivation of retinoblastoma and p53

proteins, the activation of telomerase, the activation of the proto-oncogenes c-Myc and Ras, the activation of Akt and STAT3, and the upregulation of cyclin D1. These data suggest that the key molecular pathway which are involved in oncogenesis are activated by HCMV-DB infection in HMECs [334]. Although the presence of HCMV has been shown in different malignant tissues, its importance is still a matter of debate [335]. It is not yet well understood if the virus plays a causative role in these cancers, or just simply represents an epiphenomenon [182].

1.2.11.3 Oncomodulation effect of HCMV

Cancer is a term used for diseases in which cells abnormally divide and grow and are able to invade nearby tissues. These cancer cells can spread to other parts of the body through the blood and lymph system and may results in metastatic disease which is usually incurable. As mentioned above in this thesis, several lines of evidence in cancer biology indicate that the tumorigenesis is a multistep process and several hallmarks of cancer have been identified comprising, uncontrolled and sustained cell growth, insensitivity to negative growth regulation, resistance to induced cell death, lack of senescence, genomic instability, angiogenesis and invasion and metastasis [133].

As described above, several studies indicate the presence of HCMV in different types of cancer. On other hand, different proteins which are encoded by HCMV indicate that HCMV is a complex virus even though the function of most of these proteins are still unknown [210]. Although HCMV proteins have been found in several cancer tissue specimen, it is not considered as a typical oncogenic virus [336]. The term oncomodulatory has been proposed for HCMV to describe the influence of the virus in tumorigenesis [319-321]. Oncomodulation is the ability of the virus to promote an oncogenic process characterized by disruptions in intracellular signaling pathways, transcription factors and tumor suppressor proteins in an appropriate genetic/epigenetic environment. In fact, HCMV infection leads to chromosome instability, control of the cell cycle and inducing telomerase activity, angiogenesis and cellular migration, and inhibiting apoptosis [320, 337].

It has already been reported that HCMV infection causes a rapid activation of host cell mitogen pathways, e.g. the PI3K pathway in human fibroblasts. It was also shown that PI3K activation is important for initiation of viral DNA replication, which results in cellular proliferation [338]. Mutation of one or several genes in the P13K pathways in cancer cells can lead to consistent activation of the pathway, to stimulate growth, survival and proliferation in these cells [339].

HCMV has developed multiple mechanisms to ensure activation of the MAPK in infected cells, a kinase that is known to be critical for viral infection [340]. Another study reported the role of HCMV-US28 protein (a constitutively active chemokine receptor homologue) in tumor development through induced activation of STAT-3, induction of IL-6, vascular endothelial growth factor (VEGF), and cyclooxygenase-2 (COX-2) both *in vitro* and *in vivo*

positive cells. These factors play important roles in tumor progression, angiogenesis, tumor cell migration [341, 342].

HCMV infection in the cells induce specific chromosomal breaks, p53 mutation and take control of epigenetic functions [336, 343, 344].

The HCMV IE protein can bind to p53, Rb and degrade p21 and thereby modulate cell cycle regulation and down regulate tumor suppressor proteins [345]. It has also been reported that HCMV proteins IE72, IE86 [346], pp71 [347] and UL97 [348] are involved in inactivation of the Rb tumor suppressor protein family, and promoting the cell cycle to enter S phase.

Furthermore, HCMV IE proteins activate cell survival pathways in both in normal and tumor cells trough induced expression of the transcription factor NFkB [349].

HCMV IE proteins also induce cell cycle regulation and cellular division by expressing proto-oncogenes, cyclins and kinases [350].Most notably, it was shown that HCMV can induce chromosomal break at 1q42 and 1q21 in a replication-independent manner [351]. The possible targets residing near 1q42 include the ADPRT locus associated with DNA repair and replication [352]. This deletion has been connected to the development of glioblastoma [353]

It has been proposed that HCMV has modulated multiple ways to promote angiogenesis, which is a very important component of solid tumor development [354]. Cell-free supernatants obtained from HCMV-infected cells contain higher levels of pro-angiogenic molecules and promote angiogenesis in vitro [355].

In infected glioma cells, HCMV IE induce angiogenesis either via suppression of TSP-1 expression, thrombospondin-1, anti-angiogenic factors or via induced production of IL-8 [356, 357]. It has already been shown that in HCMV infected glioblastoma cell, HCMV gB proteins bind to PDGFR-alpha which results in intracellular phosphorylation of the receptor and enhancing migration and angiogenesis [228]. Furthermore, HCMV protein can promote stemness by blocking cellular differentiation and interact with the DNA damage response pathway to alter the cell cycle [321].

1.2.12 Viral infection and epigenetic modification

DNA viruses including polyomaviruses, adenoviruses, papillomaviruses, herpesviruses, and hepatitis B virus, exist as an episome in the nucleus of the host cell. These viruses likely exploit epigenetic mechanism to regulate biological activities during their life cycle for the following reasons:

1- These viruses typically assemble into some form of nucleoprotein structure and varieties of chromatin in the nucleus, to avoid DNA damage signaling and nucleolytic attack [358].

2- As an obligate intranuclear parasite, the viruses usually use the cellular protein synthesis machinery, regulatory factors, co-factors and enzymes to accomplish gene expression and regulation.

3- These viruses require epigenetic or similar processes to allow for the different viral genome states necessary to complete an infection and to coexist with the host.

In other hand, the viruses may also epigenetically dysregulate host cell biology to enhance their own biological process, e.g. by stimulate the synthesis of factors associated with DNA replication, transcription or inhibit pathway involved in immune surveillance [359].

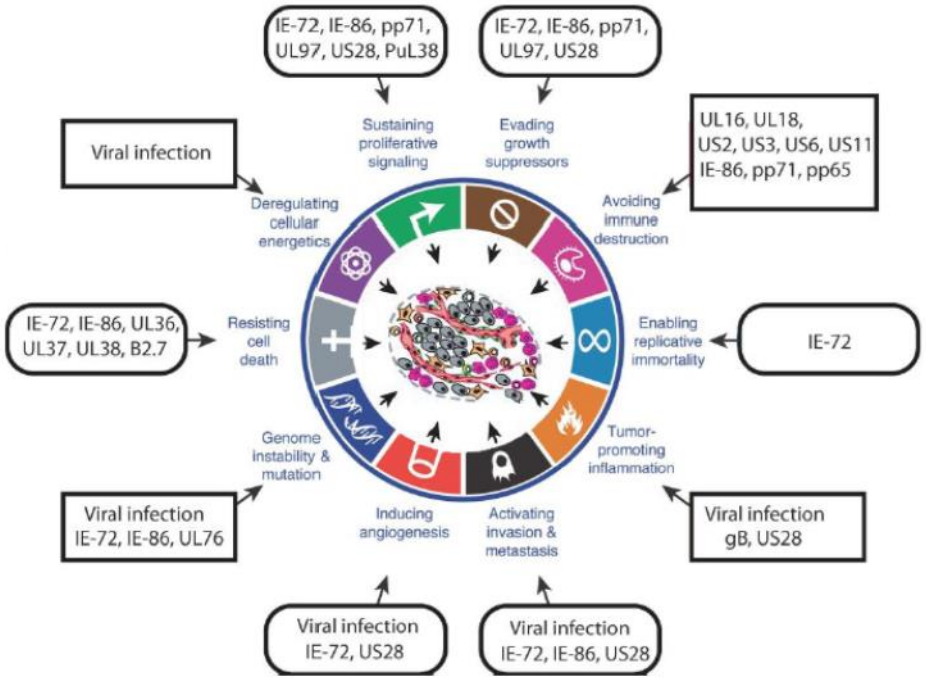

Fig 8: Hallmark of cancer, adapted from Hanahan & Weinberg, 2011

1.2.12.1 Herpesviridae and epigenetic modification

A number of the Herpesviridae family are significant human pathogens including, herpes simplex virus (HSV), Epstein–Barr virus (EBV), and Kaposi's sarcoma-associated herpesvirus (KHSV) [360]. Depending of the cellular environment, infection by herpesviruses can result in either lytic or latent infection. In lytic infection, the infected cell is ultimately killed. In contrast, in a latent infection, the virus exists as an episome in the nucleus of the infected cell, coordinate its own replication with the host, and segregate itself along with the chromosomes of the cell following replication [359]. [359]. In fact, during latency, the viral genome is in a heterochromatic state and viral transcription is suppressed. This silenced genome can be reactivated and allowing viral lytic gene expression by initiating the expression of IE genes.

This aspect of regulation is generally controlled by a viral protein, which interacts with the cellular chromosomes to tether the newly replicated virus for segregation along with the chromosome [361]. There must be a way for the virus to be in a latent phase and to reactivate and generate a lytic infection. This reactivation process is usually a result of changes in the

cellular environment and thought to contribute to epigenetic changes. During this process, a number of histone modification enzymes such as histone demethylases, plays important roles in driving IE expression even if the mechanisms involved in this process is not fully understood [361]. A recent study show that in lytic infection, histone demethylases which reverse the repressive histone H3-lysine 9 and lysine 27 methylation, are important in the activation of viral IE gene expression [361].

KSHV has been extensively studied due to its ability to cause cancer in AIDS patients. Upon infection by the KSHV virus, the chromatin structure is organized and allow for immediate-early transcription with corresponding histone modifications activating the appropriate genes and deactivating other genes [362]. Shortly after infection, the transcription factor which is involved in activating the lytic form of the virus is repressed and Latency Associated Nuclear Antigen (LANA) is produces along with a small number of other viral genes [363]. There is a gross change in chromatin structure of the virus upon reactivation [362]. There is also evidence from next-generation sequencing, showing that gene silencing in repressive chromatin may also occur through DNA methylation and interactions with noncoding RNAs in KSHV [364].

Similar to KSHV, it seems that epigenetic factors also regulate both HSV and EBV reactivation and latency. In HSV, lytic infection initiates with the chromatinization of the viral DNA, which occurs in 1-2 hours after infection. The chromatin is acted upon by transcription factors from the virus and the cell, along with chromatin remodeling factors. The chromatin modifications include changes in histone modification and nucleosome positioning resulting in immediate early gene expression. In latent infection, viral DNA is chromatinized more slowly and the genes responsible for completion of a lytic infectious cycle, appear to exist as heterochromatin with repressive histone modifications such as H3K27Me3histone modifications [358, 362-365].

As EBV plays a causing role in certain human cancers such as Burkitt´s lymphoma and post-transplant lymphoma [207], it has undergone some very extensive analyses of epigenetic modifications. The results showed certain histone modifications and gene expression or repression. For example, methylated H3K4 was located at sites of gene expression and the location of RNAPII, transcription factor [366]. In general, DNA methylation of the EBV genome, results in silencing of genes, however there are significant differences within various latency types with respect to genes silenced [367].

1.2.12.2 HCMV infection and epigenetic modification

As other DNA viruses, the HCMV genome exists as an episome in association with nucleosomes in infected cells, but during encapsidation into virions, the viral genome is believed to be completely free of histones [215, 216]. In recent years, accumulating evidence strongly suggests that the assembly and modulation of chromatin association with the viral genome is an additional layer in the regulation of viral gene replication and transcription [368].

In other words, the host cell nucleosome deposition machinery targets HCMV DNA upon infection, resulting in a stepwise and dynamic viral chromatin assembly process. This finding suggests that epigenetic events are involved in all viral DNA functions during HCMV infection such as genome replication, DNA damage response and the temporal cascade of viral gene transcription [216]. Recent studies showed the close interaction between HCMV encoded proteins and cellular epigenetic mechanism [368-370]. Indeed, previous studies have shown that post-translational chromatin remodeling is associated with the differentiation and maturation of non-permissive and un-differentiated CD34+ cells with an inactive major immediate-early promoter (MIEP) towards antigen presenting permissive cells such as dendritic cells and macrophages, resulting in reactivation of latent HCMV starting with the MIEP [371-373]. At latency, HCMV-MIEP is associated with transcriptional suppression factor (HP-1), which binds to methylated histones and at lytic phase of infection, this factor binds to acetylated histones, which is a marker of transcriptional activity [215]. Moreover, HCMV-MIEP and entire HCMV-IE locus binds to HDAC3 [374]. During reactivation of latent HCMV infection, the chromatin is remodeled and the histones are acetylated with subsequent viral gene expression [375].

In addition, another study suggested that the HCMV tegument protein, pUL97 kinase regulates viral immediate early gene expression by phosphorylation disruption of HDAC1 and binding to the IE promoter [376].

1.3 CNS TUMOR

1.3.1 - Medulloblastoma (MB)

Medulloblastomas (MB) are a compilation of molecularly diverse tumor types that originate either in the cerebellum or brain stem. Medulloblastoma of the cerebellum accounts for 20% of malignant childhood brain tumors. This type of tumor mainly occur in infancy and childhood, although it can rarely occur in adulthood. The etiology of MB is not fully understood but the impact of environmental factors such as diet, pathogens, exposure to radiation and hereditary genetic defects have previously been reported [377].

The evidence from gene expression profiling supported the existence of four main subgroups of medulloblastoma; Wnt, SHH, Group 3 and Group 4. The Wnt and SHH (Sonic Hedghog) were named for the signaling pathways that play major roles in the pathogenesis of these subgroups. The generic names for the remaining two groups were chosen since the biology underlying these subgroups were less known until relatively recently [378].

- WNT subgroup

The best known subgroup of medulloblastoma is the WNT subgroup due to its very good prognosis and long term survival [379]. Nearly all of the Wnt medulloblastomas studied to date have classic histology [380], germline mutation of the Wnt pathway and the

adenomatous polyposis coli (APC), in addition to somatic mutations of CTNNB1 encoding β-catenin, which have been found in sporadic medulloblastomas [381, 382]. TP53 mutations are identified in approximately 15% of WNT medulloblastomas but are not associated with poor prognosis [383]

- SHH subgroup

Approximately 25% of medulloblastoma occurrences are SHH subgroup tumors [384]. SHH signaling is required for normal cerebellar granule neuron precursors (CGNP) proliferation during development. Germline mutations in the Shh receptor [385], similarly, somatic mutation of SHH pathway genes such as PTCH, SMO, and SUFU as well as amplifications of downstream transcription activators, GLI1 and GLI2, have been found in this subgroup of medulloblastoma [386-388]. These mutations, causing constitutively active SHH signaling and increased downstream target gene expression, are drivers of SHH medulloblastoma [389, 390]. A part of childhood medulloblastomas contain TP53 mutations and these patients have the worst outcome although patients with adult or childhood SHH medulloblastoma with wild-type TP53 have intermediate prognosis [390, 391]. Another mutation in TERT (telomerase reverse transcriptase) promoters has been reported in SHH medulloblastoma patients, which is associated with better treatment outcomes [391-393]. MYC genes are amplified in 10% of medulloblastomas [394, 395] aberrant expression of these genes has been shown to lead to tumorigenesis [396].

The prognosis of SHH subgroup of medulloblastoma appears to be intermediate between the Wnt subgroup, with very good prognosis, and Group 3 which is poor [397-399].

- Group 3

Group 3 represents roughly 25% of medulloblastoma occurrences. In this subgroup of medulloblastomas, a number of genes that play a role in retinal development are overexpressed, although the pathogenetic effect of these genes' over expression is not currently clear [380, 400]. One subset of Group 3, Group 3α, with MYC amplifications assume the highest risk of recurrence and death. In contrast, Group 3β, another subset of Group 3 which were not found to harbor MYC amplifications, showed intermediate prognosis similar to Group 4 [401]. The terrible prognosis for Group 3 patients indicates that for this subgroup of medulloblastoma the development of practical biomarkers and understanding of the underlying pathogenesis are necessary [378].

- Group 4

The Group 4 tumors make up >30% of all medulloblastomas and present with an intermediate prognosis, similar to SHH tumors. The molecular pathogenesis of the Group 4 is not well understood [378, 380, 401]. Although an isochromosome 17q is common in Group 4, it is also seen in 26% of Group 3 tumors [380]. The only specific genetic changes among this subgroup is loss of the X chromosome, which is seen in 80% of females with Group 4 medulloblastoma. The over-representation of genes involved in neuronal differentiation and

neuronal development has been reported in Group 4, but are not yet clinically understood [380, 400, 401].

1.3.1.1 Medulloblastoma and epigenetic alteration

One of the most interesting discoveries from genomic studies of medulloblastoma is the high frequency of epigenetic regulator alterations across all four subgroups. More than 30% of medulloblastoma samples harbor mutations, deletions, or amplifications of genes encoding epigenetic regulators [402-404]. The most studied epigenetic alterations associated with human medulloblastoma are DNA methylation and histone modifications.

- DNA methylation

Alteration of DNA methylation is a common feature of tumorigenesis [14]. Analyses of genome wide DNA methylation arrays suggest that DNA methylation plays a major role in pathogenesis of MB by repressing genes associated with cell differentiation and cell death [405, 406]. Bisulfite treatment followed by PCR, showed silencing of several tumor suppressor genes by hypermethylation of CpG tumor suppressor promoters [407, 408]. Other array analysis identified aberrant methylation in some genes, such as the negative regulator of SHH signaling, inhibitors of the WNT signaling pathway, and a transcriptional repressor, among others [409]. Treatment of medulloblastoma cancer cell lines with DNA methylation inhibitors also reveal increases in expression of identified genes, although the samples used in this study were not subgroup classified [402]. Recent genome-wide studies of DNA methylation patterns in subgroup classified medulloblastoma have provided new insights into the tumor molecular pathogenesis. By using methylation microarray, DNA methylation status at 1505 loci in 807 genes was analyzed in 230 medulloblastoma samples which were previously classified with transcriptome profiles. The data showed that the four subgroups identified based on DNA methylation are highly related to their transcriptome counterparts. This finding suggests that each medulloblastoma subgroup has a specific DNA methylation pattern which can be used as a robust sub-classification standard [410]. It has already been reported that an oncogene VAV1 is hypomethylated with elevated expression in most human SHH medulloblastoma and mouse SHH medulloblastoma models [411]. Another study determined the high expression of DNMT1, DNMT3A and DNMT3B in human medulloblastoma samples, suggesting that promoter hypermethylation may play a role in medulloblastoma development [412]. Notably, DNA methylation in tumors can be inherited from their cell-of-origin neural lineages, but can also be generated de novo in tumors. It is not quite clear if these cancer-specific DNA methylation alterations are regulated by the activities of DNA methyltransferases and/or enzymes that remove the methyl group, such as TET enzymes [406].

- Histone acetylation

Histone modifications including, acetylation, methylation, phosphorylation, and ubiquitination may either alter local chromatin structures or, more importantly, recruit proteins that recognize the modifications and regulate gene transcription. In

medulloblastoma, multiple genes including histone modification enzymes are altered [388]. A comprehensive study which analysed more than 1000 medulloblastoma samples, confirmed the importance of copy number changes of histone modification in medulloblastoma. Recurrent focal amplifications and homozygous deletions in genes targeting histone lysine methylation, particularly that of H3K9, were identified in medulloblastoma [413]. The mechanism(s) behind these epigenetic changes the impact on medulloblastoma development are still unclear [406]. Group 3 and Group 4 medulloblastomas have relatively high global H3K27me3 levels [414]. A global high H3K4me3 and low H3K27me3 status is associated with better prognosis than low levels of both H3K4me3 and H3K27me3 within Group 3 and Group 4 tumors [403].

In addition, histone acetyltransferases (HATs) and histone deacetylases (HDACs) also have important roles in medulloblastoma. It is reported that hMOF, a HAT enzyme, is down regulated in many medulloblastoma samples, and this down regulation is associated with low survival [415]. The HAT genes are also mutated in some medulloblastoma patients [414, 416].

In general, HDACs likely support medulloblastoma growth, and HDAC inhibitors alone or in combination with other inhibitors are being tested in clinical trials for treatment of medulloblastoma [417-419].

1.3.1.2 Medulloblastoma and HCMV

Recent studies indicate that HCMV immediate-early and late proteins are expressed in the majority of primary medulloblastomas. In addition, this study showed that the anti-viral drug, ganciclovir significantly reduces tumor growth in mice carrying established human medulloblastoma xenografts [196].

Another study from the same group demonstrated that HCMV was detected in 92% medulloblastoma. They also showed that engrafted cells containing HCMV nucleic acids in nude mice exhibited induced HCMV protein expression, which was correlated with COX-2 expression in primary tumor cells, cell lines and medulloblastoma xenografts. Most importantly, the combination of the anti-viral drug valgancicovir and the COX-2 inhibitor, celecoxib prevented HCMV replication in vitro leading to reduced production of PGE2 and decreased tumor growth [196].

It has been shown that HCMV may impact the medulloblastoma host cell replication stress and DNA repair [420]. Others showed that HCMV encoded protein US28 promotes cell proliferation, angiogenesis and cell cycle progression, which can represent an oncomodulatory role of HCMV in medulloblastoma through induced activation of STAT-3, induction of IL-6, vascular endothelial growth factor (VEGF), and cyclooxygenase-2 (COX-2) in US28 positive cells. [421, 422].

1.3.1.3 Treatment of medulloblastoma

The current treatment for patients with this highly malignant tumor consists of surgery, whole brain and spinal cord radiation (in patients above 3 years), and aggressive chemotherapy, sometimes followed by stem cell transplantation [413] [406]. Even though the overall survival of medulloblastoma patients is relatively high (long-term survival (>5 years) of MB patients is 60% - 70%) the patients often experience disease and complications related to treatment including developmental, neurological and psychological deficits [423] [406]

Current treatment for medulloblastoma patients > 3 years of age is based on radiation to the brain and spine with a boost to the tumor bed, together with combinations of multidrug chemotherapy [423, 424]. A major goal for the children < 3 years of age is to eliminate or greatly reduce the radiation to the brain and spine due to the brain development in these early age of life. The treatment can consist of surgery dependent on the size of the tumor, and stem cell transplantation [425, 426]. High dose chemotherapy in young children and infants without irradiation gives nearly 75% overall 5-year survival for favorable subgroups [427]. Under such circumstances, a new effective treatments with minimal damage to the developing brain in these children is highly relevant.

Recent comprehensive epigenetic analyses have revealed a deeper understanding of the mechanisms of MB pathogenesis [405, 414, 428] and inhibition of DNMTs may result in a decreased tumor formation. Therefore, DNMTs could be served as valuable targets for designing of specific antitumor agents [429].

1.3.2 Glioblastoma (GBM)

Glioblastoma is an aggressive central nervous system (CNS) malignancy with a median survival of 15 months despite significant progress in surgical resection, chemo and radiotherapy [430] [431]. The risk factors for GBM are unknown but heredity, environmental exposures to radiation, vinyl chloride and pesticides have been considered in the initiation of GBM [1, 432, 433]. Even traumatic brain injury involving inflammation has been suggested in the etiology of GBM, although this is still controversial [434]. Additionally, HCMV infections are considered to be a common risk factor and part of the pathogenesis, although this is still controversial.

1.3.2.1 Glioblastoma and epigenetic alteration

Besides genetic abnormality, aberrant epigenetic alterations also contribute to GBM [435]. Three subgroups of GBM show alteration in both DNA methylation and methylation of lysine residue of histone proteins [35, 436]. DNA methylation is however, the most studied epigenetic alteration in GBM.

- DNA methylation

Epigenomic profiling has revealed aberrant DNA methylation mediates in GBM development and malignancy [437]. Epigenetic silencing through promoter hypermethylation of the O6-

methylguanine methyltransferase (MGMT) gene has been widely described in glioma. MGMT is an important repair enzyme which contributes to resistance to temozolomide, a chemotherapy using for treatment of GBM patient. MGMT promoter methylation silences this gene which results in decreasing DNA repair activity and increasing the susceptibility of the tumor cells to temozolomide [438-440]. It is reported that the promoter of several genes involved in key cellular functions such as the cell cycle [441], tumor suppression [442], DNA repair [443], tumor invasion [444] and apoptosis [445], have been silenced in association with promoter hypermethylation in malignant glioblastoma. One single study reported a significant overexpression of DNMT1 and DNMT3B in 20 cases of GBMs. This study proposed that the overexpression of DNMT1 and DNMT3B in gliomas leads to hypermethylation of various tumor suppressor genes, resulting in lack of growth regulation and higher genome instability, resulting in poor prognosis in gliomas. This research also showed that the DNMT1 gene is differentially regulated by both histone acetylation and histone methylation in gliomas. It is evident from this study that histone modifications apart from methylation, play a pivotal role in DNMT1 gene expression. [446].

Furthermore, the link between the 1p/19q co-deletion and epigenetic alterations in the course of demethylation/hypomethylation has previously been studied and shown a positive association with IDH promoter mutations resulting in production of the oncometabolite R-2-hydroxyglutarate. Different enzymes, including the epigenetic regulator TET2 enzymes, are inhibited by R-2-hydroxyglutarate causing demethylation/ hypermethylation of DNA [447, 448], specifically, hydroxylation of 5-methylcytosine (5mC) to generate 5-hydroxymethylcytosine (5hmC) [447].

The Cancer Genome Atlas (TCGA) research network performed whole genome sequencing of GBM tumors and found that GBM recurrence is linked to epigenetic mechanisms and pathways [449]. A next generation sequencing analysis revealed a global decrease in H3K4me3 in GBM, especially at promoters and CpG islands [450].

1.3.2.2 Glioblastoma and HCMV

HCMV has been detected in 90-100% of GBMs [1] [451] [328]. Similar to medulloblastoma, HCMV nucleic acids and proteins were found in majority of patients with high and low grade of gliomas. In several studies, the expression of HCMV early and late gene products have already reported in these tumors [1, 7, 196]. HCMV DNA has also been detected in peripheral blood of glioma patients [451]. A recent study showed that low grade HCMV infection in GBM tumors was highly associated with survival longer than 18 months [328]. Although the exact role of HCMV in GBM is still under investigation, evidence suggest that HCMV plays an oncomodulatory role in GBM and can disrupt certain pathways involved in the cell cycle, apoptosis and angiogenesis [452, 453].

Additionally, the positive effect of antiviral treatment by valganciclovir (Valcyte) in glioblastoma patients has been reported. Tumor growth was restricted and survival was longer in patients treated with ganciclovir [454]. More than 250 glioblastoma patient were

examined and only one of them was HCMV negative. From the 75 patients evaluated in that study, the median rate of overall survival was 33 months in those with low-grade HCMV infection and 13 months in those with high-grade HCMV infection, while the median rates of 2-year survival were 63.6% and 17.2%, respectively [454].

1.3.2.3 Treatment of Glioblastoma

The current standard treatment for GBM is resection, radiation, and chemotherapy. Due to the highly invasive nature of GBM cells, malignant gliomas cannot be completely eliminated surgically [455]. The current combination treatment regime includes the alkylating agent temozolomide (TMZ) and radiation [430].

As mentioned before, O6-methylguanine methyltransferase (MGMT) is an important repair enzyme which contributes to resistance to temozolomide. MGMT promoter methylation silences this gene and results to decreasing DNA repair activity and increasing the susceptibility of the tumor cells to temozolomide. Glioblastoma patients with MGMT methylated promoter who were treated with temozolomide had longer overall survival of 21.7 months. In contrast, patients without MGMT promoter methylation who were treated with temozolomide had a significantly shorter median survival [456]. However, TMZ is used in the treatment of glioblastomas regardless of MGMT promoter methylation status [455]. In addition, GBM cells develop resistance against the current treatment regimen that includes TMZ and radiation. [457, 458]. Despite decades of research and treatment, the median survival of GBM patients still remains at 15 months [431]. Due to the difficulties in treatment of GBM patients, new strategies that effectively target GBM cells and/or overcome their resistance to treatment will be necessary [455]. As DNA methylation is a reversible epigenetic phenomena and due to overexpression of DNA methyltransferases in gliomas, DNMTs may serve as a marker for cancer cells and may be an important target in the treatment of glioblastoma [446, 459].

2 AIM OF THE STUDY

The aim of this thesis were:

1- To study the impact of HDAC inhibitor on genomic DNA methylation changes.

2- To investigate the mechanism(s) involved in cellular relocalization of DNMTs following HCMV infection.

3- To study the effect of HCMV infection on host cell DNA methylation in malignant CNS tumor.

4- To determine the role of HCMV for epigenetic alteration in GBM and MB.

3 RESULTS AND DISCUSSION

3.1 STUDY I

Genomic DNA hypomethylation by histone deacetylase inhibition implicates DNMT1 nuclear dynamics

- Background

Histone deacetylase inhibitors (HDACi) are considered as antitumor drugs acting through e.g. reactivation of silenced tumor suppressor genes. Several HDACi have been developed and are currently in clinical trials both for hematological and solid tissue malignancies. Accumulating evidence has shown that combination of HDACi and DNA methylation inhibitors (DNMTi) represents a promising cancer therapy strategy. There is some evidence that synergistic effects of HDACi and DNMTi is achieved by their action on common targets, including DNA methyltransferase 1 (DNMT1). Our study aimed to investigate further the interaction between HDACi, DNMTi and DNA global DNA methylation. We studied the effect of the HDACi, Trichostatin A (TSA) on global and gene-specific DNA methylation and applied methods with single molecule sensitivity, confocal laser scanning microscopy and fluorescence correlation spectroscopy (FCS), to study TSA effects on the nuclear dynamics of DNMT1 in live cells.

- Results and discussion

Our study confirmed that TSA causes genomic hypomethylation and involves a decrease of nuclear DNMT1 protein levels. By using FCS, we have also shown that both the DNMT inhibitor 5AZA and the HDAC inhibitor TSA change the nuclear kinetics of DNMT1. The mechanisms behind the TSA effects are apparently different from the mechanisms of action of 5AZA. These data show that the effects of HDACi are not limited to direct hyperacetylation of histones but may indirectly affect other epigenetic factors, such as DNMT1 activity, through converging pathways. An increased knowledge about the interaction between epigenetic modifiers on nuclear kinetics will be important for therapy applications using epigenetic drugs. Our study thus sheds light on the molecular mechanisms underlying the synergistic action of HDACi and DNMTi and may also provide a basis for defining improved policies for cancer treatment using combined epigenetic therapy.

3.2 STUDY II

Human cytomegalovirus infection is sensitive to the host cell DNA methylation state and alters global DNA methylation capacity

- Background

Human Cytomegalovirus (HCMV) is a ubiquitous herpesvirus that infects and establishes latency in the majority of the human population, and may cause fatal infections in immunocompromised patients. Recent data showed a close interaction between HCMV

encoded proteins and cellular epigenetic mechanisms such as histone acetylation and deacetylation. An important strategy for viruses should be to change host cell epigenetic regulatory systems in favour of the viral functions. This strategy would provide the opportunity for the virus to impair host cell protective mechanisms and consequently replicate its genome and spread.

In this study, we examined interactions between HCMV infection and DNA methylation machinery in different host cells.

- Results and discussion

Our data demonstrated that:

1- DNA methylation influences cellular susceptibility to HCMV infection.

2- Human cytomegalovirus inhibits the host cell DNA methylation machinery.

3- Infection of human fibroblasts by HCMV results in re-localization of DNMTs from the nucleus to cytoplasm of the cell.

In conclusion, HCMV infection results in profound effects on the host cell DNA methylation machinery and is associated with inflammation in vivo. Our results improved the understanding of cytomegalovirus pathogenesis and may disclose new targets for antiviral therapy. These findings may also contribute to the further understanding of mechanisms involved in DNA methylation abnormalities in physiological and pathological conditions.

3.3 STUDY III

Increased cytomegalovirus replication by 5-Azacytidine and viral-induced cytoplasmic expression of DNMT1 in medulloblastoma and endothelial cells

- Background

Medulloblastomas (MBs) are the most common pediatric brain tumors and associated with a poor prognosis. The etiology of MB is not fully understood, yet the impact of epigenetic alterations of oncogenes has previously been established. During the past decade, the human cytomegalovirus (HCMV) has been detected in several types of cancer, including MB. Since DNA methylation occurs in the cell nucleus and this is, besides being intimately involved in gene regulation, also considered a host defense response against invading nucleic acids, we studied the impact of HCMV infection on DNA methyltransferase (DNMT1) in MB (D324) cells, human umbilical vein endothelial cells (HUVECs) as well as in MB tissue sections. We hypothesized that infection and DNMT1 intracellular localization are linked.

- Results and discussion

Our results determined that DNMT1 localized to the nucleus of uninfected and HCMV-IE expressing D324 cells and HUVECs, but accumulated in the extra nuclear space in all

HCMV-gB positive cells. Inhibition of HCMV late protein expression by Cymevene® (ganciclovir) prevented the cytoplasmic localization of DNMT1. Treatment of HCMV infected D324 cells and HUVECs with the methylation inhibitor 5AZA, significantly increased HCMV-IE and late HCMV-gB gene transcription and protein expression. Immunohistochemical staining of DNMT1 and HCMV proteins in MB cancer tissue sections revealed both nuclear and cytoplasmic DNMT1 localization. In conclusion, DNMT1 resides in the cytoplasm of HCMV-gB expressing HUVECs and D324 cells. Increased viral protein synthesis in 5AZA treated cells suggests that HCMV replication may benefit from a DNA methyltransferase-free cellular environment. Our findings emphasize the importance of assessing potential viral activation in the treatment of MB patients with epigenetic drugs.

3.4 STUDY IV

Nuclear exclusion of DNA methyltransferase-1, and reduced invasion by 5-azacytidine, in human cytomegalovirus infected glioblastoma cells

- Background

Glioblastoma (GBM) is the most aggressive brain tumor in the adult, with a devastating outcome. Despite aggressive surgery and advanced therapy, the median overall survival of these patients is only approximately 14 months. The risk factors for GBM are not well understood. Emerging reports support the presence of HCMV proteins and nucleic acids in GBM tissues. Furthermore, accumulating data strongly support an association between histone modification, chromatin modulation, HCMV genome transcription and replication, both at latency and during lytic infection. DNA methylation is important for initiation and progression of cancer and is an established host response against invading nucleic acids such as viruses. We have previously reported increased HCMV replication in DNA methylation inhibited non-tumor (HUVEC) and medulloblastoma cells.

In this study, we investigated the viral replication, proliferation and invasion capacity of 5AZA treated HCMV infected GBM cells (U343) and examined the expression of DNMT1 and HCMV proteins in GBM tissue specimens.

- Results and discussion

We observed DNMT1 to be localized in the nucleus of cells expressing HCMV-IE but coincided with an extra-nuclear/cytoplasmic localization in U343 cells expressing the HCMV-gB protein. In tissue specimens, DNMT1 was expressed in the nucleus of tumor cells, but localized to the extra-nuclear/cytoplasmic space of cells lining blood vessel walls within the GBM tumors. 5AZA treatment of HCMV infected U343 cells did not affect viral replication but attenuated the invasion and proliferation ability of these cells. While 5AZA treatment of uninfected U343 cells did not affect invasion ability, proliferation was significantly reduced.

DNMT1 is localized to the extra nuclear space of U343 cells expressing HCMV-gB proteins and in the cells of blood vessel wall within GBM tumors. 5AZA treatment of U343 cells leads to reduced proliferation of uninfected and HCMV infected cells while HCMV infected cells are vulnerable to 5AZA treatment, leading to a decreased invasion.

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